IDENTIFICATION OF FILIFACTOR ALOCIS IN PERIODONTAL BIOFILMS USING POLYMERASE CHAIN REACTION TECHNIQUE – A CROSS SECTIONAL STUDY

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partial fulfilment of the requirements
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MASTER OF DENTAL SURGERY

BRANCH – II
PERIODONTICS

THE TAMIL NADU DR. M.G.R. MEDICAL UNIVERSITY
Chennai – 600 032

2015 - 2018

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This is to certify that the Dissertation entitled **"IDENTIFICATION OF FILIFACTOR ALOCIS IN PERIODONTAL BIOFILMS USING POLYMERASE CHAIN REACTION TECHNIQUE – A CROSS SECTIONAL STUDY"** is a bonafide work done by **Dr. K. POORANA**, Post Graduate student (2015–2018) in the Department of Periodontics, under the guidance of **Dr. MAHEASWARI RAJENDRAN**, Professor, Department of Periodontics, Tamil Nadu Government Dental College and Hospital, Chennai – 600 003.

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I hereby declare that this dissertation titled **"IDENTIFICATION OF FILIFACTOR ALOCIS IN PERIODONTAL BIOFILMS USING POLYMERASE CHAIN REACTION TECHNIQUE – A CROSS SECTIONAL STUDY"** is a bonafide and genuine research work carried out by me under the guidance of **Dr. MAHEASWARI RAJENDRAN, M.D.S., Professor and Guide,** Department of Periodontics, Tamil Nadu Government Dental College and Hospital, Chennai - 600003.

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This agreement herein after the "Agreement" is entered into on this day ----------between the Tamil Nadu Government Dental College and Hospital represented by its **Principal** having address at Tamil Nadu Government Dental College and Hospital, Chennai – 600 003, (hereafter referred to as, 'the college')

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Dr. K. POORANA, aged 40 years currently studying as **Post Graduate student** in Department of Periodontics, Tamil Nadu Government Dental College and Hospital, Chennai – 600 003 (herein after referred to as the 'PG student and Principal Investigator'),

And

Mrs. Dr. MAHEASWARI RAJENDRAN, M.D.S., aged 52 years working as **Professor** in Department of Periodontics at the Tamil Nadu Government Dental College and Hospital, Chennai – 600 003 (hereafter referred to as 'Co-investigator'),

Whereas the PG student as part of her curriculum undertakes this research on **"IDENTIFICATION OF FILIFACTOR ALOCIS IN PERIODONTAL BIOFILMS USING POLYMERASE CHAIN REACTION TECHNIQUE – A CROSS SECTIONAL STUDY"** for which purpose the Co–investigator and the college shall provide the requisite infrastructure based on availability and also provide facility to the PG student as to the extent possible as a Principal Investigator

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date : 02-08-2016

Sub: IEC review of the research proposals,

Title of the work: Identification of Filifactor Alocis in periodontal biofilms using polymerase chain reaction technique–A cross sectional study

Principal Investigator: Dr. K. Poorana
II yr pg student.

Department : Department of Periodontics
Tamil Nadu Govt. Dental College & Hospital , Chennai-3

Thank you for submitting your research proposal , which was considered at the Institutional Ethics Committee meeting held on the 01.07. 2016, at TN Govt. Dental College and the documents related to the study referred above were discussed and the modifications done as suggested and reported to us through your letter dated 01-08-2016 have been reviewed. The decision of the members of the committee , the secretary and the Chairperson IEC of TN Govt. Dental College is here under:

Approved	Approved and advised to proceed with the study
Approved with suggestions	----------
Revision	----------

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ABSTRACT

Background:

Periodontitis is a disease of tooth supporting tissues and is inflammatory in origin. The etiological role of bacteria is established, although contributions of individual species or groups of organisms are unclear and unrecognized periodontal pathogens remain to be identified. A strong association has emerged between newly identified gram positive *Filifactor alocis* and periodontitis. Polymerase Chain Reaction (PCR) is a highly sensitive and specific technique for the microbiological analysis of the subgingival plaque.

Aim:

To identify the presence of *Filifactor alocis* in periodontal biofilms through PCR technique in healthy, generalized chronic periodontitis and generalized aggressive periodontitis subjects.

Materials and methods:

In this study, subgingival plaque samples were obtained from 45 subjects divided into three groups based on their periodontal conditions namely healthy n=15, generalized chronic periodontitis (GCP) n=15, and generalized aggressive periodontitis (GAP) n=15. The clinical parameters, plaque index, sulcus bleeding index, probing pocket depth and clinical attachment level were determined. Furthermore, *Filifactor alocis* was identified using Real-time PCR.

Results:

In **t**he selected site, the difference between mean plaque index, sulcus bleeding index and clinical attachment level were statistically significant between healthy and periodontitis (chronic and aggressive) sites. The mean probing pocket depth of healthy group was 2.71±0.22, 5.76±0.58 for chronic periodontitis, and 6.27±0.14 for aggressive periodontitis which was statistically significant ($p<0.05$). *Filifactor alocis* was identified in all 45 samples and it was expressed in terms of cycle threshold (CT) values and was found to be higher in deep diseased pockets rather than healthy. The mean CT value of healthy control group was 38.01, chronic periodontitis group was 30.85 and aggressive periodontitis group was 28.92.

Conclusion:

Within the limits of present study, it can be concluded that *Filifactor alocis* is a potent diagnostic marker organism for periodontal disease and it should be considered an important periodontal pathogen. The putative pathogens cause dysbiosis and the role of putative periodontal pathogen *F. alocis* causing dysbiosis need to be evaluated in future studies for preventive, predictive and personalized treatment, beneficial for patients.

Key words: Filifactor alocis, PCR, chronic periodontitis, aggressive periodontitis

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LIST OF ABBREVIATIONS

A. actinomycetemcomitans	*Aggregatibacter actinomycetemcomitans*
AAGPRs	Asaccharolytic Anaerobic Gram-Positive Rods
ANOVA	Analysis of Variance
BANA	N-Benzoyl-DL-Arginine-2Napthylamide
CAL	Clinical Attachment Level
CEJ	Cemento–Enamel Junction
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CT	Cycle Threshold
DNA	Deoxyribo Nucleic Acid
D. pneumosintes	*Dialister pneumosintes*
E. corrodens	*Eikenella corrodens*
E. nodatum	*Eubacterium nodatum*
F. alocis	*Filifactor alocis*
GAP	Generalized Aggressive Periodontitis
GCP	Generalized Chronic Periodontitis
IL	Interleukin
mRNA	messenger Ribo Nucleic Acid

MSCRAMMs	Microbial Surface Components Recognizing Adhesive Matrix Molecules
PCR	Polymerase Chain Reaction
P. gingivalis	*Porphyromonas gingivalis*
P. intermedia	*Prevotella intermedia*
PPD	Probing Pocket Depth
PSD	Polymicrobial Synergy and Dysbiosis
qPCR	Quantitative Polymerase Chain Reaction
RFLP	Restricted Fragment Length Polymorphism
RT-PCR	Real-time Polymerase Chain Reaction
RNA	Ribo Nucleic Acid
ROS	Reactive Oxygen Species
rRNA	ribosomal Ribo Nucleic Acid
SD	Standard Deviation
SPSS	Statistical Package for Social Science
T. denticola	*Treponema denticola*
T. forsythia	*Tannerella forsythia*
Th	T helper
TNF	Tumour Necrosis Factor

Introduction

INTRODUCTION

Periodontitis is a biofilm-induced chronic inflammatory disease which leads to the annihilation of the periodontium [***Darveau, 2010***][1]. The tissue damage in periodontitis is umpired by disparaging host immune responses coordinated by a team of periodontal pathogens in subgingival biofilm [***Haffajee, Socransky, 1994***][2]. Although the etiological role of bacteria is proven, impacts of individual species or clusters of organisms are vague.

There is meagre data to consider that, definitely cultivable bacteria bestow more to the pathogenesis of periodontitis than fastidious microbes. It is also unclear that the recognized periodontal pathogens *P. gingivalis*, *P. intermedia* and *T. forsythia* are apt diagnostic markers to differentiate between health and disease [***Kumar et al, 2006, Riep et al, 2009***][3,4] as greater than 50% of the phylotypes documented in the oral cavity are yet-to-be cultured [***Dewhirst et al, 2010, Griffen et al, 2012***][5,6]. This raises queries on the relative significance of these organisms in the etiopathogenesis of periodontal disease.

The dogma that gram negative microbes preponderate is no longer plausible with the substantial escalation of gram positive microbes in deep unhealthy sites. As documented by some studies, gram positive organisms are found in larger quantity than gram negative organisms. *Filifactor alocis (F. alocis*) is an example for such an organism.

F. alocis (ATCC 35896T) a fastidious, gram-positive, obligately anaerobic rod, was first isolated in 1985 in human gingival sulcus [**Cato et al, 1985**][7]. A robust relationship has developed between *F. alocis* and oral diseases, exclusively periodontitis.

F.alocis is attributed as the second most prevalent in chronic periodontitis and third most prevalent in generalized aggressive periodontitis and proposed to be an excellent marker organism for periodontal disease [***Schlafer et al, 2010***][8]. A study by Dahlén and Leonhardt concluded that, *F.alocis* should be added to the 12 species used for routine diagnostics of periodontitis-associated bacterial flora [***Dahlén and Leonhardt, 2006***][9]. Kumar et al, in 2005, proposed *F.alocis* as a potential marker for periodontal disease activity, since it was found in high numbers in chronic periodontitis patients with periodontal destruction when compared with healthy subjects [***Kumar et al, 2005***][10].

Markers of periodontal disease activity are studied through numerous diagnostic methodologies. By monitoring the presence or absence of pathogens in a patient, one can predict the recurrence of disease and also can improve the prognosis. The "gold standard" culture methods to detect periodontal pathogens have inherent advantages and also numerous limitations.

The evolutionary technique, Polymerase Chain Reaction (PCR) invented by Mullis, overwhelms the limitations and is an extremely sensitive and specific technique for exploration of subgingival plaque biofilm [***Eick and Pfister, 2002***][11]. Real-time Polymerase Chain Reaction (RT-PCR) permits unswerving observation of the cumulative quantity of PCR products by way of the enzymatic assay in the subjected sample.

In the existing study, the polymerase chain reaction technique is chosen to explore the existence of *F.alocis* in subjects with generalized chronic periodontitis (GCP), generalized aggressive periodontitis (GAP), and healthy control group.

Aim and Objectives

AIM AND OBJECTIVES

AIM:

To identify the presence of *Filifactor alocis* in periodontal biofilms through real-time polymerase chain reaction technique in healthy, generalized chronic periodontitis and generalized aggressive periodontitis subjects.

OBJECTIVES:

The objective of the present study was to develop a molecular biology approach for determining the presence of *Filifactor alocis* in healthy, generalized chronic periodontitis and generalized aggressive periodontitis.

Review of Literature

REVIEW OF LITERATURE

Periodontitis, characterized by chronic inflammation, alveolar bone loss and destruction of gingival and periodontal ligament attachments to the teeth, affects 10-15% of adult populations worldwide ***(Petersen and Ogawa, 2012)***[12]. With more than 650 species of bacteria identified in the human oral cavity, only a subset of these microbes which now includes previously unrecognized and yet-unculturable species [***Belstrom et al, 2014***][13] are associated with the disease.

The microbial aetiology of periodontal disease has been the focus of researchers for a long time. With about 400 species detected in the gingival sulcus and some of them viz. *Porphyromonas gingivalis* and *Tannerella forsythia* widely regarded as major pathogens and fulfill the criteria for candidate pathogen. [***Kumar et al, 2005***][14]

In the initial era, microbial search for periodontal pathogens was confined to culture based methods and relied heavily on artificial media. The advent of deoxyribonucleic acid (DNA) based strategies has changed the scenario. This molecular based search while confirming the presence of already identified organisms, the presence of several species, which were not detectable by prior culture based techniques remained unanswered.

The novel and path-breaking ribosomal 16S cloning and sequencing has facilitated identification of several hitherto uncultivable bacteria in the oral cavity. Based on 16S cloning and sequencing of human subgingival flora, Paster et al demonstrated that 40% bacterial species present to be novel species or phylotypes [***Paster et al, 2001***][15]

The microbial role in periodontal pathogenesis although is increasingly realized, nothing substantive has emerged. Socransky in 1998, appropriately defined the microbial aetiology of periodontal disease with an appealing statement "specific bacteria of right clonal type with essential genetic elements in numbers for that host with appropriate additional species in the right environment." [***Socransky et al, 1998***][16]

He also organized the organisms into complexes which "simplified" the description of multiple organisms of uncertain role into selected and few complexes with specific relationship to periodontal health and disease [***Haffajee et al, 2008***][17]

Periodontal microbiota is more heterogeneous than earlier believed, with 700 organism believed to exist, 200 present in any one individual and about 50 present at any one site [***Aas et al, 2005***][18]. While the prime role in aetiology is devoted to red complex organisms, little has been known about other organisms, which also are reported to be associated with periodontal disease. Several unexplained situations need to be addressed. Red complex organism like *P. gingivalis* can be found in the absence of disease, precluding its role as an exogenous pathogen. [***Ximénez-Fyvie et al, 2000***] [19]

With the plethora of the organism and their uncertain role, Hajishengallis and Lamont in an invited review introduced the spirit of polymicrobial synergy and dysbiosis model (PSD) of periodontal disease. The model revolves around certain species, termed "keystone pathogens," to modulate host response in ways that impair immune surveillance and tip the balance from homeostasis to dysbiosis. [***Hajishengallis et al, 2012***] [20]

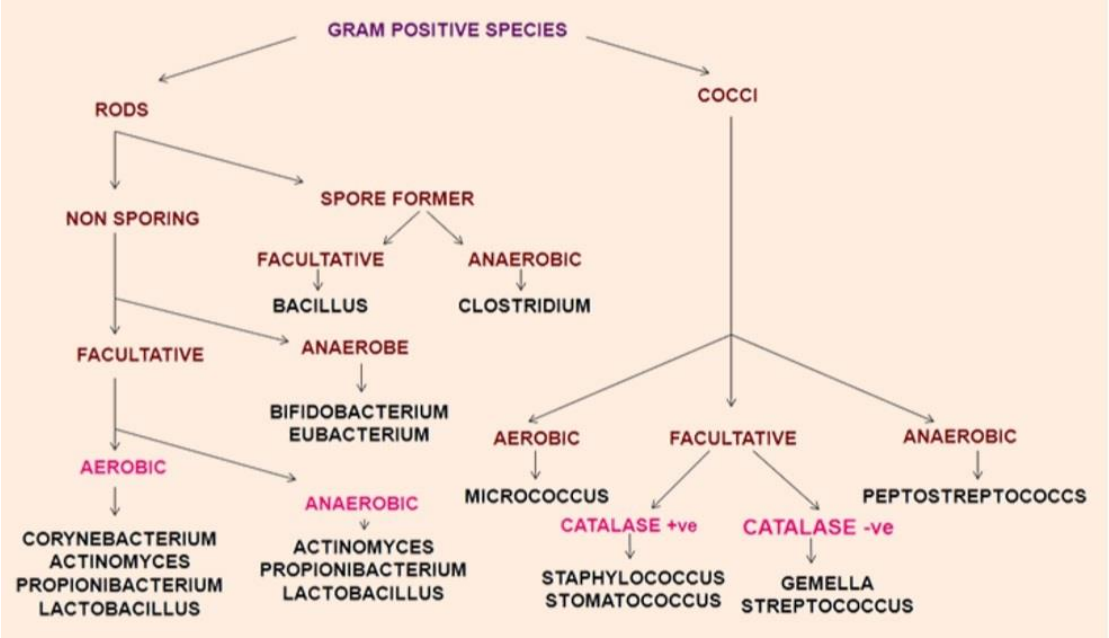
The following are essentially associated with periodontitis: *P. gingivalis, T. forsythia, Aggregatibacter actinomycetemcomitans, Prevotella intermedia, Prevotella melaninogenica, Fusobacterium nucleatum, Parvimonas micra, Eikenella corrodens, Prevotella nigrescens, Capnocytophaga gingivalis, Treponema denticola, Treponema socranskii, Eubacterium nodatum* and *Campylobacter rectus* [***Teles et al, 2013***][21]

The following organisms have also been implicated as periodontal pathogens: *Porphyromonas endodontalis, Prevotella denticola, Filifactor alocis, Selenomonas, Synergistes, Desulfobulbus, TM7 (*new candidate bacterial division*) Cryptobacterium curtum, Eubacterium saphenum, Mogibacterium timidum, Prevotella corporis, Prevotella disiens, Peptostreptococcus magnus, Slackia exigua, Treponema maltophilum, Treponema sp. Smibert-3, Treponema lecithinolyticum, Treponema putidum sp. nov., Enterococcus faecalis, Escherichia coli* and *Bartonella sp.* [***Teles et al, 2013***] [21]

And, the list of putative pathogens is growing, and now includes a very diverse group of microorganisms in both the Bacteria and Archaea domains. It is becoming increasingly apparent that periodontal infections are caused by a much more diverse microbiota than merely gram-negative anaerobes. Nevertheless, the relevance of certain species as etiological agents of periodontal diseases remains controversial, even among microbiologists.

Gram-Positive Bacteria in Periodontal Disease

Classification system of bacteria discovered by Gram in 1884, allows a large proportion of clinically important bacteria to be classified as either Gram positive or negative. The following flow chart depicts the classification system of Gram positive species in detail as given by **Gita et al., 2016.**



The potentially pathogenic role of bacteria, which were not considered as primary keystone pathogens in periodontal disease, has been implicated in periodontal literature time and again. Paul Keyes, way back in 1970, said - "I am convinced that although many clinicians and investigators do not exclude the role of bacteria in periodontal lesions, at this point interest in microorganisms often dissipates and attention shifts to other areas".

Gram-positive anaerobes have been isolated way back in 1990s from periodontal biofilms [***Rams et al, 1992***][22]. Kumar et al, also identified the dominance of Gram positive anaerobic species in periodontally diseased sites compared to healthy individuals [***Kumar et al., 2005***][14]. In another study supporting this evidence, Haffajee & Socransky, examined supragingival biofilm samples to understand the nature of the microbial complexes that exist in supra-gingival plaque. [***Haffajee and Socransky, 2005***][23]

An interesting observation was that *Eubacterium nodatum*, a Gram-positive anaerobe was found both in the mature and the long-term redevelopment biofilms along with the red complex species, *P. gingivalis, T. forsythia*, and *T. denticola* usually observed in subgingival plaque. Not surprisingly the same research group while studying the subgingival samples found merit in including *E. nodatum* as a part of the red complex.

Apart from the established organisms, Gram-positive anaerobes such as *Filifactor alocis, Peptostreptococcus micros* and *Eubacterium nodatum* have been recently isolated from patients with periodontitis, and are emerging to be considered as important contributors to the bacterial aetiology of periodontitis. However, the literature evidence is controversial and inconsistent across various studies. This discrepancy could be explained by geographic variability [***Nasidze et al, 2009***][24], or by difference in the depths of the pockets sampled [***Socransky and Haffajee, 1998***][25], as well as the sample size and DNA analytic bias. **[*Dahlen and Leonhardt, 2006*][9]**

Among these gram positive organisms, *Filifactor alocis* (*F.alocis*) has gained attention in the past decade due to growing evidence of its potential role in pathogenesis of periodontitis of various forms.

Periodontitis

The different forms of periodontitis are chronic periodontitis, aggressive periodontitis, and as a manifestation of systemic disease **(*Armitage, 1999*)**[26]. The most common form of periodontitis is the chronic periodontitis, and is most prevalent among adults, rarely observed in children.

Chronic periodontitis is associated with the accumulation of plaque and calculus; it generally has a slow to moderate rate of disease progression, but periods of more rapid destruction may also be observed. Increases in the rate of disease progression may be caused by the impact of local, systemic, or environmental factors.

Aggressive periodontitis generally affects systemically healthy individuals less than 30 years of age, though patients may be older. It is distinguished from chronic periodontitis by the age of onset, the rapid rate of destruction, familial aggregation of diseased individuals, and a strong racial influence.

The microbiology of chronic and aggressive periodontitis though varies, some pathogens do commonly occur in both types of periodontitis.

Filifactor Alocis

Filifactor alocis (F. Alocis) is a non-spore forming, gram-positive obligate anaerobic rod that is slow growing and generally unreactive to conventional biochemical tests, hence difficult to identify [***Jalava, Eerola, 1999***][27]. The main habitat of *F. alocis* is the gingival sulcus [***Cato et al, 1985***][8]. *F. alocis* was first isolated in 1985 from the gingival sulcus in gingivitis and periodontitis patients and originally classified as *Fusobacterium alocis*, [***Cato et al, 1985***][8] but later reclassified into the genus *Filifactor [**Jalava, Eerola, 1999***][27].

In silico analysis of *F. alocis* has shown close relatedness to *Clostridium* and *Fusobacterium* [***Aruni et al, 2011***][28]. Common to these genera is their asaccharolytic nature thus an ability to utilize specific amino acids including arginine. Consistent with this observation, arginine has been shown to stimulate the growth of *F. alocis* [***Uematsu et al, 2003***][29].

F. alocis has been acknowledged in patients with chronic periodontitis (CP) by Kumar et al in 2003 and Dahlen and Leonhardt in 2006 and in patients with generalized aggressive periodontitis (GAP) by Hutter et al in 2003 [***Kumar et al, 2003, Dahlen and Leonhardt, 2006, Hutter et al, 2003***][10, 9,30].

Siqueira and Rocas, in 2003, identified the organism in endodontic infections [***Siqueira and Rocas, 2003***][31]. There is documented evidence that *F. alocis* is associated with peri-implantitis. Tamura et al. have shown that the sulcus around oral implants with peri-implantitis harbours high levels of asaccharolytic anaerobic Gram-positive rods (AAGPRs) including *F. alocis*, which is one of the most prominent in that environment [***Tamura et al, 2013***][32]

Virulence Factors

F. alocis appears to have unique properties such as resistance to oxidative stress with its stimulated growth under this condition and genes coding for a well-developed amino acid metabolic pathway that can allow it to colonize and survive with other traditional periodontal pathogens in the stress environment of the periodontal pocket [***Aruni et al, 2011, 2014***][28,33].

These unique properties of *F. alocis* in addition to its ability to interact with other microbial species forming a polymicrobial synergistic relationship can enhance its invasive capacity [***Aruni et al, 2011***][28] and cause chronic inflammation [***Fine et al, 2013***][34] in prevailing adverse conditions including fluctuations in nutrient availability, temperature, pH and oxygen tension.

Additionally, an impact of *F. alocis* on the host is its ability to induce proinflammatory cytokines triggering apoptosis of gingival epithelial cells [***Moffatt et al, 2011***][35].

Proteases

Proteases play a significant role in virulence among the major oral pathogens. The *F. alocis* genome possesses at least 15 different proteases [***Aruni et al, 2012***][36].

Some of the membrane bound proteases of *F. alocis*

- Caax protease - involved in protein and/or peptide modification and secretion [***Pei et al, 2001***][37].
- Xaa-pro-dipeptidase
- O-sialoendo-peptidase
- Nlp/P60 family protein (peptidase M23/37)
- Oligo endo-peptidase M3 family [***Kumagai et al, 2005***][38]

O Chioma et al, demonstrated that the recombinant *F. alocis* peptidase U32 protein (designated PrtFAC) can interact with, and degrade type I collagen, heat denatured collagen and gelatin in a calcium dependent manner. PrtFAC decreased viability and induced apoptosis of normal oral keratinocytes (NOKs) in a time and dose-dependent manner. [***O Chioma et al, 2016***][39]

Adhesion

Attributes such as adherence and invasion of host cells, are considered important to the success of a pathogen. *F. alocis* was shown to adhere and invade epithelial cells, enhanced in the presence of *P. gingivalis* [***Aruni et al, 2011***] [28].

Vesicle mediated internalization of *P. gingivalis* and *F. alocis* was observed during invasion of epithelial cells in co-infection studies [***Aruni et al, 2011***] [28]. This process may protect the pathogen after invasion, facilitating its pathogenic potential.

The community dynamics through the interaction of *F. alocis* and *P. gingivalis* may result in the upregulation of specific factor(s) that may enhance their virulence potential [***Aruni et al, 2011***] [28].

MSCRAMMs

Proteomic analysis of *F. alocis* during co-infection of epithelial cells with *P. gingivalis* using tandem mass tagging technique revealed increase in several membrane adhesion proteins and Microbial Surface Components Recognizing Adhesive Matrix Molecules (MSCRAMMs)

MSCARMMs are evidently important in the pathogen attachment and virulence modulation. Expression of *F. alocis* MSCRAMMs have been identified in the cell membrane and cell wall fractions [***Aruni et al, 2012***][36].

Unique amino acid metabolism

Even though *F. alocis* showed low gingipain-type activity, it had increased non-gingipain protease activity [***Aruni et al, 2011***] [28]. The amino acids mostly utilized by *F. alocis* include arginine and lysine, followed by cysteine. The *F. alocis* arginine metabolic pathway predicts the enzymatic degradation of arginine by arginine deiminase, leading to the conversion of arginine to ornithine and ammonia [***Uematsu et al***] [29].

Arginine degradation could favour increase in the pH that would counteract acidic conditions generated from carbohydrate catabolism in a mixed bacterial oral flora. In the periodontal pocket, these amino acids can also be made available from the degradation of various protein substrates by other bacteria and host-derived proteases for nutritional support, survival and virulence [***Eley et al, 1982***][40].

The occurrence of a wide range of dipeptidases, metalloproteases and o-sialoglycoproteases, could likely provide *F. alocis* with the appropriate substrates to compensate for its nutritional needs. Additionally, certain proteins, such as the oxy acyl carrier protein, fibronectin binding protein and dipicolinate reductase which are involved in amino acid metabolism and virulence [***Berges et al, 1986***][41], were also identified in *F. alocis* [***Aruni et al, 2012***] [36].

Co-existence, polymicrobial synergy and biofilm formation

Biofilm formation involving *F. alocis* has been demonstrated both in periodontic and endodontic cases [***Schlafer et al, 2010***][42]. *F. alocis* is likely to interact with a variety of oral bacteria and participate in community development forming heterotypic communities with *P. gingivalis* [***Wang et al, 2013***] [43].

Hui Chen et al, 2015, identified *F. alocis*-centered co-occurrence group of potential pathogens namely *P. gingivalis*, *P. endodontalis*, *T. forsythia*, *E. nodatum*, *Fretibacterium sp., Lachnospiraceae [G-8] sp. and Peptostreptococcaceae* [XI][G-4] sp., in the human oral cavity that was significantly enriched in periodontitis samples. [***Hui Chen et al, 2015***][44]. These results laid a microbial ecological foundation for future etiological studies in periodontitis.

Oxidative stress resistance

F. alocis is relatively resistant to oxidative stress and that its growth is stimulated under those conditions [***Aruni et al, 2011***][28]. These observations may indicate an important attribute for the survival and relative abundance of *F. alocis* compared to other organisms in the inflammatory microenvironment of the periodontal pocket.

CRISPR-associated genes

Regions of unusual DNA composition in the bacterial genome such as the clustered regularly interspaced short palindromic repeats (CRISPR) locus together with the CRISPR-associated genes are thought to act as adaptive immunity systems in bacteria and recently found to play a role in bacterial virulence [***Louwen et al, 2014***][45]. The virulence attribute may partly be due to genome rearrangement and/or regulation of gene expression that will facilitate host environment adaptation.

CRISPRs are widely distributed amongst bacteria and archaea [***Chylinski et al, 2014***][46] and show some sequence similarities [***Kunin et al, 2007***][47]. The upregulation of CRISPR/Cas system components during the co-infection of epithelial cells with *F. alocis* and *P. gingivalis* could suggest their role in virulence and pathogen synergy. Most of the potential virulence attributes of this assacharolytic bacteria is unexplored and deserves further extensive study [***Aruni et al., 2015***][48]

Diagnosis of periodontal disease

The traditional diagnostic methods described in the following sections aim to identify the etiologic factors, assess the clinical signs of the inflammatory process, and determine the degree to which periodontal destruction has occurred.

Assessment of Etiologic Factors

Bacteria are the initiating factor and primary target of the majority of the present therapeutic modalities. It is also imperative to identify any factor that make an individual susceptible to the build-up of dental plaque. The factors include a lack of manual dexterity associated with arthritis or other conditions, improper technique, a reduced frequency of oral hygiene practices and tooth anatomy that promotes plaque retention.

Susceptibility to periodontitis is increased by several established risk factors including diabetes mellitus and cigarette smoking **(*Papapanou PN, 1996*)**[49]. Genetic syndromes are also associated with periodontal disease **(*Kinane, 2005*)**[50]. It is for these reasons that a thorough medical history, as well as a history of cigarette smoking, is an essential part of establishing a periodontal diagnosis.

Assessment of Gingival Inflammation

A great deal of information regarding the degree of gingival inflammation could be obtained by a simple visual inspection of the tissues. The healthy gingiva is typically pink in colour, firm in consistency and has a knife-edged margin. Inflamed tissues reveal cardinal signs of inflammation, such as redness and swelling.

Bleeding on probing is an important marker of gingival inflammation within the periodontal pocket. It occurs due to the presence of micro ulcerations in the junctional epithelium and is influenced by repeated probe insertions within a short time and by the use of excessive force (>25 N).

Purulent exudate is also an important sign of gingival inflammation; but, true suppuration may perhaps be difficult to distinguish from the plaque expressed from the gingival crevice [***Wolf, Lamster, 2011***][51].

Assessment of Loss of Periodontal Attachment

Probing Pocket Depth (PPD) assessment is probably the most commonly used clinical measure for detecting loss of periodontal support. It is measured from the free gingival margin to the depth of the probable crevice. The depth of a healthy gingival sulcus ranges from 1 to 3mm. PPD is not the most objective measure of loss of periodontal tissues because the position of the free gingival margin is variable.

When there is gingival inflammation, the free gingival margin may be located more coronal than normal because of oedema in the tissues. In this condition, there may be a deeper-than-normal PPD even in the absence of loss of periodontal attachment. Such a deepened pocket is known as a pseudo pocket.

True periodontal pocket can occur due to apical migration of the junctional epithelium and loss of supporting tissues of the tooth. The PPD may also be normal in the presence of significant attachment loss. This might occur in the case of treated periodontitis or when the disease progression manifests with gingival recession rather than pocket formation.

Clinical Attachment Loss (CAL) is a more objective measure of the loss of periodontal support because it is calculated from a fixed point on the tooth, usually the cemento-enamel junction (CEJ), if detectable. But existing CAL does not give any indication of current disease activity. [***Wolf, Lamster, 2011***] [51]

Radiographic assessment of periodontal disease

Radiographs are necessary component of the periodontal examination and crucial in establishing a periodontal diagnosis. It gives important information about the position and architecture of the alveolar crest of bone. Despite their importance in periodontal diagnosis, radiographs have numerous limitations as diagnostic tools such as they do not give any information about disease activity or progression and tend to underestimate the amount of attachment loss [***Akesson L et al., 1992***] [52] and that clinical changes (attachment loss) come before radiographic changes [***Goodson JM et al., 1984***] [53].

Supplemental diagnostic tests

The clinical and radiographic assessments described before are the most commonly used measures of periodontal disease. But, there are several supplemental tests including microbial, biochemical, and genetic tests that have been developed to address the fact that traditional approaches do not adequately identify patients or sites with progressive disease (or at risk for progressive disease). Supplemental tests can also be used to assess the response to treatment and determine appropriate recall intervals [***Armitage GC, 2003***] [54].

Microbial sampling:

The collection of subgingival plaque samples is the common way for the determination of periodontopathic bacteria. There are many way of collection of subgingival plaque. The commonly used sampling devices were discussed by ***Tanner and Goodson, 1986*** [55].

They described the sampling tools as "dental approved" devices. Sampling using curettes, scalers, paper points, barbed broaches within cannulas, irrigation of periodontal pockets etc. were reported. A 10 µl automatic pipette was used for sampling in a study of ***Strand et al., 1987*** [56].

Another method called pocket-out-method based on collecting biological materials of non-viable periodontal pathogens originating from the pocket was described by ***Smola et al., 2003***[57]. ***Tanner and Goodson, 1986*** [55] stated that paper points are used by an increasing number of investigators mostly for cultural studies; hereby the loosely adherent tissue associated microorganisms in the periodontal pocket were sampled.

Loomer, 2004 [58] reported that paper point samples differ from curette samples and that curette collects plaque from the entire pocket whereas paper points collect plaque from the outer layer of the plaque, which contains more pathogens. In another study by ***Renvert et al., 1992*** [59] paper point samples were compared with scaler samples both before and after treatment. This study concluded that paper points collected more colony forming units and spirochetes before and after therapy.

Microbial tests:

Dark field and phase contrast microscopy was the first method used to demonstrate plaque microorganisms [***Rosebury et al, 1950***][60]. But an exact quantitative testing of plaque samples with such a technique is not possible. The information about the vitality of the plaque is limited. Only motile bacteria can be distinguished as alive [***Listgarten, 1986, Lange et al. 1983, Müller et al., 1989***][61, 62,63]

Another identification method is bacterial cultivation, which represents the golden standard in microbiological diagnosis, although it possesses some disadvantages. Target microorganisms must survive sampling and transportation and stay vital in order to be able to colonize. Several putative periodontopathogenic microorganisms require anaerobic growth conditions. Therefore, for diagnostic procedures problems of sampling, transport and cultivation have to be taken into consideration. Limitations with respect to detecting non-viable bacteria, the inability of some species to grow reliably on selective media as well as high costs narrow the use in periodontal microbiological diagnostics [***Loomer, 2004***] [58].

Enzyme tests are very fast methods and mostly used as a chair side diagnostic. As a reaction between the microbiological enzymes and the testing agent occurs, the colour of the containing medium changes to reflect the detection of microorganisms. The BANA test (N-Benzoyl-DL-Arginine-2Napthylamide) is an example for this method which detects microbial groups rather than species and does not give quantitative results [***Jervøe-Storm, 1992, Loomer, 2004, Loesche, 1986***] [64, 58, 65].

The evolution of more advanced molecular diagnostic techniques like polymerase chain reaction (PCR) in the early 1980s, has revolutionised the field of microbiology. PCR based methods are more specific and sensitive than cultivation, based on detection of gene specific DNA sequences, thus a possibility to distinguish close related bacteria is given.

History of Polymerase Chain Reaction:

The field of human genetics started on when DNA was first isolated by Johann Friedrich Miescher in 1869 **[*Dahm, 2005*] [66]**. Watson and Crick in 1953 described the double helix structure of DNA **[*Dahm, 2005*] [66]**. In 1975, Southern blotting technology was used for genetic analysis. Its adaptation RFLP was developed in 1980 by Ray White [***Tilstone et al., 2006***] **[67]**.

One of the most important revolutionary techniques in molecular biology, the PCR was introduced by Kary Mullis et al. in the 1983 and he won the Noble prize in Chemistry in 1993 for its discovery. They developed it as a rapid and two times sensitive procedure than standard Southern blotting for the detection of the sickle cell mutation which is the first application of PCR in the field of medicine.

This molecular technique, invented three decades ago, now has revolutionized various fields. In dentistry, as early as 1992, PCR was used to identify DNA from human tooth pulp tissue for use in forensic dentistry.

Various derivatives of conventional PCR including nested PCR, multiplex-PCR, reverse transcriptase PCR, allele-specific PCR and quantitative PCR (qPCR) or real-time PCR (RT-PCR) subsequently evolved playing significant role in the field of Periodontology **[*Leys et al., 1994, Riggio et al., 1996, Saygun et al., 2002, Kobayashi et al., 2000, Lyons et al., 2000*] [68, 69, 70, 71, 72]**. In 2005, open-ended PCRs were used for genome mapping of the entire bacterial spectrum in the plaque sample [***Kumar et al., 2005***] **[3]**.

Standard PCR lacks the ability to quantify target DNA molecules because only an endpoint determination can be analysed. RT-PCR overcomes these problems by direct monitoring of the increasing amount of PCR products throughout the enzymatic assay. The data for quantification are collected in exponential phases of the PCR. This allows a precise quantification of the target DNA copy number, when using internal and external standards [***Bustin, 2000***]**[73]**

Principles of Polymerase Chain Reaction:

PCR, an *in vitro* technique, allow amplification and study of the genes and their RNA transcripts obtained from various tissue sources including peripheral blood, skin, saliva, gingival crevicular fluid, semen and hair **[*Jordan et al., 2001, Jervoe-Storm et al., 2005*] [74, 64]** .

Each assay requires the presence of template DNA, primers, nucleotides, and DNA polymerase. Template DNA is the identified target sequence that needs to be amplified and it range from 100 to 1000 base pairs in length. Primers are short, single-stranded sequences of nucleic acid (oligonucleotides) selected to specifically anneal to a particular nucleic acid target **[*Tille, 2013*]**[75]. Primer pairs containing forward and reverse primer, each 16 to 20 base pairs in length are used **[*Turgeon 2011*] [76]**.

DNA polymerase is the DNA replicating enzyme that links individual nucleotides together to form the PCR product and thus to amplify target sequences of DNA.

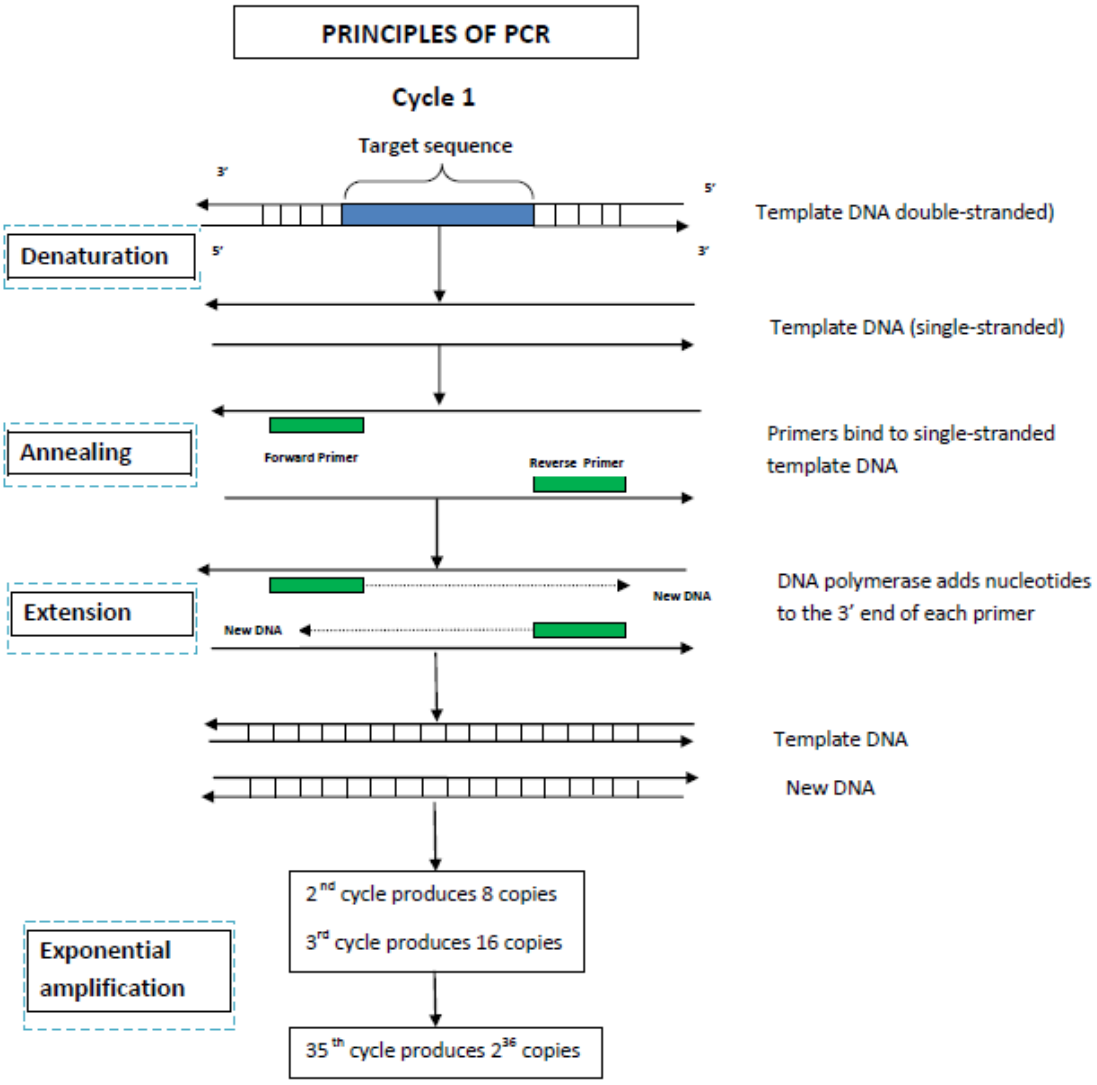
Nucleic acid is first extracted from the clinical sample by heat, enzymatic or chemical methods. Once it is extracted, target nucleic acid is added to the reaction mix containing primers, components to optimize polymerase activity (i.e., buffer, cation [$MgCl_2$], salts and deoxynucleotides) and enzymes in a test tube or 96-well plate and then placed in a thermal cycler that allows repeated cycles of DNA amplification to occur in the following three basic steps

1. DNA denaturation – Separation of the double DNA strands into two single strands is accomplished by heating to 94ºC.
2. Primary annealing – At 50º C to 58º C, when the primer pair is mixed with the denatured target DNA, forward primer anneals to a specific site at one end of the target sequence of one target strand, plus the reverse primer anneals to a specific site at the opposite end of other complementary target strand.
3. Extension of the primed DNA sequence – The enzyme DNA polymerase synthesizes new complementary strands by the extension of primers at 72 ºC **[*Turgeon, 2011*] [76]**.

Automated programmable thermal cyclers carry the PCR mixture through each reaction step at the precise temperature and for an optimal duration.

The process is repeated 30 times in general. At the finish of 30 cycles, the reaction mixture contains about 2^{30} molecules of the desired product [***Turgeon, 2011***]**[76]**. Once amplification reactions have occurred, a variety of manual and automated methods are there to detect the amplified product **[*Tille, 2013*][75]**.

Principle of PCR:



Applications of PCR in Dentistry:

PCR plays an important role in various fields of dentistry. The subgingival plaque, saliva, mouth wash, blood, gingival tissue and buccal mucosa scraping are used in the PCR to identify microorganisms, mRNA gene expression of various inflammatory mediators and genetic polymorphisms in dentistry [***Jervoe-Storm 2005, Kumar et al., 2010, Boutaga et al., 2007, Garlet et al., 2006, Figuero et al., 2014, Garcia-Delaney et al., 2015***] [64, 77, 78, 79, 80, 81].

The knowledge of the ecology of the oral cavity has been well understood using PCR studies [***Bizzarro et al., 2013***] [82]. Epidemiological studies based on the microbiology of dental diseases, their relation to systemic diseases and genetic polymorphisms can be established.

Dental caries pathogens can be identified by PCR and it also explains the progress of dental caries **[*Okada et al., 2002*]** [83]. The microorganisms responsible for endodontic infections can be identified **[*Bogen and Slots, 1999, Kim et al., 2002*]** [84, 85]**.** Genetic markers for oral cancers are identified by the PCR technique and they are used in diagnosing and predicting the outcome and response to treatment **[*Murdoch-Kinch 1999*]** [86].

Identification of microbial pathogens:

Putative perio-pathogens including *P. gingivalis, A. actinomycetemcomitans, P. intermedia, T. forsythia, T. denticola, Prevotella nigrescens, Parvimonas micra, Eubacteria, Campylobacter rectus, Capnocytophaga sputigena, Capnocytophaga ochracea, and Capnocytophaga gingivalis* have been detected in subgingival plaque samples **[*Lyons at al., 2000, Jervoe-Storm et al., 2005, Hayashi et al., 2001, Sencimen et al., 2014, Oettinger-Barak et al., 2014, Bringuier et al., 2013*]** [64, 72, 87, 88. 89, 90]**.**

The PCR technique is a more accurate, sensitive, and rapid technique in the detection, identification and quantification of periodontal bacteria [***Eick and Pfister 2002, Riggio et al., 1996, Morillo et al., 2003***] [11, 91, 92].

Qian Wang et al, 2014, studied *F. alocis* infection and inflammatory responses in the mouse subcutaneous chamber model using fluorescence in situ hybridization and RT-PCR. **[*Qian Wang et al, 2014*] [93]**

Taqman q-PCR assays were used to determine the absolute and relative counts of *P. gingivalis, T. forsythia, T. denticola, P. micra, F. alocis, oral Synergistetes* and oral TM7s by Al-hebshi et al. [***Al-hebshi et al, 2014*] [94].** A PCR assay for the identification of *P. gingivalis, A. actinomycetemcomitans, P. intermedia, T. forsythia* and *T. denticola* has been described [***Eick and Pfister 2002, Jervoe Storm et al., 2005*] [11, 72].**

Schlafer et al, 2010, used PCR and subsequent dot blot hybridization to compare the prevalence of *F. alocis* in subgingival samples of patients suffering from generalized aggressive periodontitis (GAP), chronic periodontitis (CP), and control subjects resistant to periodontitis.[***Schlafer et al, 2010*] [39]**

Kumar et al., using open-ended PCR/sequencing techniques detected gram positive organisms *Peptostreptococcus* and *Filifactor*, genera *Megasphaera* and *Desulfobulbus*, species or phylotypes of *Atopobium*, *Campylobacter*, *Catonella*, *Dialister*, *Deferribacteres*, *Eubacterium*, *Streptococcus*, *Selenomonas*, *Treponema*, and *Tannerella* which were elevated in periodontal disease.[***Kumar et al., 2005*] [3]**

Ghayoumi et al., using rRNA PCR identification method determined the presence of *D. pneumosintes* from periodontal pockets and implicated it as "candidate pathogen". Presence of this organism in about 83% of patients with severe periodontitis and in only 19% of patients with slight periodontitis lead to the implication of the organism being considered as "suspected periodontal pathogens" **[*Ghayoumi et al., 2002*][95]**

Since the bacterial pathogens are not solely responsible for the development of periodontitis, PCR is used for research purposes to determine the prevalence of various viruses. Herpes Simplex Virus (HSV), Human Papilloma Virus (HPV), HIV, Human Cytomegalovirus (HCMV), and Epstein-Barr Virus Type I and II (EBV 1 and 2) in the gingival crevicular fluid of the individuals could be isolated in various forms of periodontal disease [***Saygun et al., 2002., Parra and Slots 1996., Pucar et al., 2007***]**[70,96,97]**.

PCR is also used to study the association of the systemic diseases such as coronary heart disease, chronic kidney disease, pregnancy complications, diabetes, respiratory disease and osteoporosis with periodontitis by identifying the periodontal pathogen levels in various tissue samples such as subgingival plaque, thrombi, coronary artherosclerotic plaque, carotid endarterectomy, placenta, aortic valves, and maxillary sinus tissue/wash samples.

Diagnostic tests such as the MicroDent® Test, ParoCheck® kits, MyPerioPath® Test and oralDNA® using multiplex PCR scheme are commercially available to assess the microbiota in subgingival plaque samples and they provide crucial information for a prevention strategy for healthy patients and treatment plans for "at risk" patients **(Do et al., 2013)[98].**

Materials and Methods

MATERIALS AND METHODS

Study population:

A total of 45 subjects comprising three groups of 15 subjects in each group were included in this study, from the out-patient ward of the Department of Periodontics, Tamil Nadu Government Dental College and Hospital, Chennai-600003. Three groups of subjects were healthy, generalized chronic periodontitis (GCP) and generalized aggressive periodontitis (GAP). Subjects from both the sex within the age range of 20 - 50 years were included. Informed consent was obtained from each subject prior to enrolment in the study.

Eligibility criteria:

Inclusion Criteria are as follows:-

- Systemically healthy subjects
- Subjects with at least 20 remaining natural teeth with at least five teeth in each quadrant.
- Healthy group - Subjects with PPD ≤ 3 mm
- Generalized chronic periodontitis - A minimum of two sites per quadrant with probing pocket depth ≥ 4mm
- Generalized aggressive periodontitis
    - Subjects $\leq$ 25 years - a minimum of two interproximal sites per quadrant with probing pocket depth ≥ 4 mm
    - Subjects 26 - 35 years - a minimum of two interproximal sites per quadrant with probing pocket depth ≥ 6 mm

Exclusion Criteria are as follows:-

- Subjects who are suffering from any known systemic diseases or immunocompromised
- Subjects who had received any surgical or non-surgical therapy six months prior to the start of the study
- Subjects who had received any antibiotic therapy in the last six months
- Pregnancy and lactation
- Subjects with habit of betel-nut, pan masala, tobacco chewing, smoking and alcohol consumption

Study Design:

- Ethical clearances were obtained from the Institution's Ethical Committee and the ethical principles were meticulously followed throughout the course of the study.
- Subjects for the study were selected if they satisfied the inclusion criteria, with no discrimination on the basis of sex, caste, religion or socioeconomic status.
- A total of 45 patients were selected for the study.
- After explaining the study procedure, written informed consent was obtained from all the subjects selected for the study.
- The study was of cross-sectional type.
- A thorough medical and dental history of the subjects was taken.
- Both clinical evaluation and microbiological evaluation was done.

Study protocol:

- The institutional ethical committee approval
- Medical History and Informed Consent
- Complete intra oral evaluation
- Periodontal Examination using clinical parameters namely Sulcus Bleeding Index, Plaque Index, Probing Pocket Depth and Clinical Attachment Level.
- Selected site analysis including clinical evaluation and subgingival plaque sampling for microbial analysis
- Clinical photographs

Armamentarium:

For clinical examination:

- Mouth mirror
- William's periodontal probe
- Curved explorer
- Dental tweezers
- Kidney tray
- Bowls
- Cotton roll
- Gauze

- Surgical gloves
- Disposable facemask
- Disposable headcap
- Patient apron

For subgingival plaque sample collection:

- Mouth mirror
- William's periodontal probe
- Curved explorer
- Dental tweezers
- Kidney tray
- Cotton roll
- Curette
- Nitrile gloves
- Paper points No. 30
- Microcentrifuge tube

Clinical parameters:

Plaque index *(Silness and Loe, 1964)*

Teeth examined – All teeth

Surfaces examined – 4 sites for each tooth (Disto-facial, Facial, Mesio-facial, Lingual/Palatal)

Criteria for Scoring

Score 0 – No plaque in the gingival area.

Score 1 – A film of plaque adhering to the free gingival margin and adjacent area of the tooth. The plaque may only be recognized by running a probe across the tooth surface.

Score 2 – Moderate accumulation of soft deposits within the gingival pocket, on the gingival margin and/or adjacent tooth surface, which can be seen by the naked eye.

Score 3 – Abundance of soft matter within the gingival pocket and/or on the gingival margin and adjacent tooth surface.

Calculation

Plaque index for a tooth = Total score from 4 areas of each tooth/ 4

Plaque index for the individual = Total Plaque index of each tooth / Number of teeth examined

Interpretation of Plaque index score

0 – Excellent oral hygiene

0.1 to 0.9 – Good oral hygiene

1.0 to 1.9 – Fair oral hygiene

2.0 to 3.0 – Poor oral hygiene

Sulcus bleeding index *(Muhlemann and Son, 1971)*

Sulcus Bleeding Index is performed through gentle probing of the orifice of the gingival crevice. Bleeding after probing to the base of the probable pocket is recorded.

Criteria for Scoring

Score 0 – gingiva of normal texture and color, no bleeding;

Score 1 – gingiva apparently normal, bleeding on probing;

Score 2 – bleeding on probing, change in color, no edema;

Score 3 – bleeding on probing, change in color, slight edema

Score 4 – either:

a) bleeding on probing, change in color, obvious edema

b) bleeding on probing, obvious edema

Score 5 – bleeding on probing and spontaneous bleeding, change in color, marked edema

Calculation

Sulcus Bleeding index for a tooth = Total score from 4 areas of each tooth/ 4

Sulcus Bleeding index for the individual = Total Sulcus Bleeding index of each tooth / Number of teeth examined

Probing Pocket Depth

Probing Pocket Depth was measured in millimeter (mm) from the gingival margin to the base of the pocket using William's Periodontal Probe. The probe was passed within the gingival sulcus along the circumference of the tooth.

Three measurements were made on the buccal aspect and three on lingual aspect of each tooth – total of six sites per tooth (Mesiobuccal, Midbuccal, Distobuccal, Mesiolingual, Midlingual, Distolingual).

Clinical Attachment Level

Clinical Attachment Level was measured from the Cemento–Enamel Junction (CEJ) to the base of the pocket using William's Periodontal Probe.

- When the gingival margin was located on the anatomic crown, the level of the attachment was determined by subtracting from the probing pocket depth, the distance from the gingival margin to the CEJ. If both were the same, the loss of attachment was calculated to be zero.
- When the gingival margin coincided with the CEJ, the loss of attachment was calculated as equaling the probing pocket depth.
- When the gingival margin was located apical to the CEJ, the loss of attachment was greater than the probing pocket depth and therefore the distance between the CEJ and the gingival margin were added to the PPD.

Three measurements were made on the buccal aspect and three on the lingual aspect of each tooth – total of six sites per tooth (Mesiobuccal, Midbuccal, Distobuccal, Mesiolingual, Midlingual, and Distolingual).

Sampling for Real-time PCR Microbial Analysis:

- Subgingival plaque sample was collected from the tooth of selected site analysis.
- Tooth was isolated with cotton rolls, supragingival plaque was removed using sterile curette.
- Subgingival plaque sample was taken by the introduction of two sterile paper points (Densply®), number 30, inserted into the depth of the pocket.
- Thirty seconds after placement in the pocket, paper points were immediately inserted in a micro- centrifuge tube and kept over ice and transported to the microbiological laboratory for downstream analysis.

Microbial analysis of subgingival plaque samples:

Microbial counts of *Filifactor alocis* in the collected samples was assessed by analyzing the expression of 16S rRNA gene sequences by real-time polymerase chain reaction (RT-PCR) technique at the Central Research Facility, Sri Ramachandra University, Chennai.

DNA isolation

Lysozyme enzyme solution was prepared containing Tris HCl, EDTA, tritonex, lysozyme (20 mg per sample). To the subgingival plaque samples, 1 mL of the above prepared solution was added and incubated at 37°C for 30 min in a water bath. Next, 20 μL of 10% sodium dodecyl sulfate was added to the sample and again incubated at 37°C for 30 min in the bath. After incubation, an equal volume of phenol chloroform solution was added and centrifuged at 10,000 rpm for 10 min.

The supernatant was collected and transferred into new Eppendorf tubes, Chloro-iso-amine alcohol of equal volume was added and the samples were centrifuged at 10,000 rpm for 10 min. The supernatant was removed and transferred into a new Eppendorf tube, 1/10th the volume of sodium acetate and 300 μL of 100% ethanol (absolute) was added and stored at -50º C overnight. The following day, the samples were centrifuged at 10,000 rpm for 10 min. The supernatant was removed and discarded and the pellet was air dried. A total of 30-50 μL of distilled water were added (RNA/DNAase-free water).

Primers:

The primer sequences for *Filifactor alocis* as suggested by ***Siqueira and Rocas, 2004***, are provided below:

Target gene	**Primer sequences (5'-3')** **F –Forward; R – Reverse**	**Annealing Temp.**	**Product Size**
Filifactor alocis	F: 5′-CAGGTGGTTTAACAAGTTAGTGG-3′ R: 5′-CTAAGTTGTCCTTAGCTGTCTCG-3′	95°C	310 bp

Real-Time PCR:

Technique:

The reaction mix was prepared to a final volume of 20 µL and loaded in an optical 96 well plate, which was then covered with an optical adhesive sheet. *F. alocis* was identified using PCR amplification of 16S rRNA gene sequences. The primers specific for *F. alocis* 16S rRNA: forward, 5'-CAGGTGGTTTAACAAGTTAGTGG-3'; reverse, 5'-CTAAGTTGTCCTTAGCTGTCTCG-3' were used.

The PCR amplicon length was 310 bp. The samples were run in duplicate. RT-PCR was performed using a thermal cycler (7900 HT RTPCR, Applied Biosystem, UK) with SYBR green fluorophore. Reactions were run in a total volume of 20 µL including 10 µL of SYBR Green Supermix, 1 µL of each primer at 10 µM concentrations, 2 µL of DNA template and 6 µL of sterile distilled water.

Reaction mix:

S.No	**Contents**	**Volume(µl)**
1	SYBR Green mix (2x)	10
2	Forward primer	1
3	Reverse primer	1
4	Template	1
5	Sterile water	7
	Total	**20**

Thermocycling conditions were 50°C for 2 min (denaturation) and then at 95ºC for 10 min to activate. The amplification was then repeated 40 times (95°C for 15 seconds) and extension at 60°C for 1 min. Negative controls without cDNA were also performed (Negative Template Control). A melting curve analysis was made after each run to ensure a single amplified product for every reaction. The results analyzed with the RQ manager (CT calculated)

Real Time Standard Program:

Steps	**Temperature (in Deg Celcius)**	**Time**
Denaturation	50°C	2 min
Annealing	95°C 95 °C	10 min 15 sec
Extension	60°C	1min
Repeat	40 cycles	

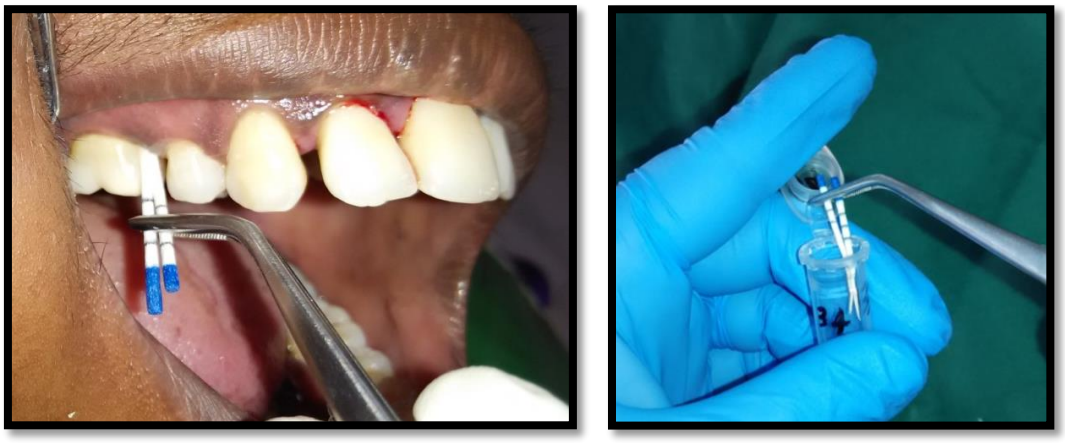
Photograph 1: Armamentarium for clinical examination

Photograph 2: Measurement of probing pocket depth

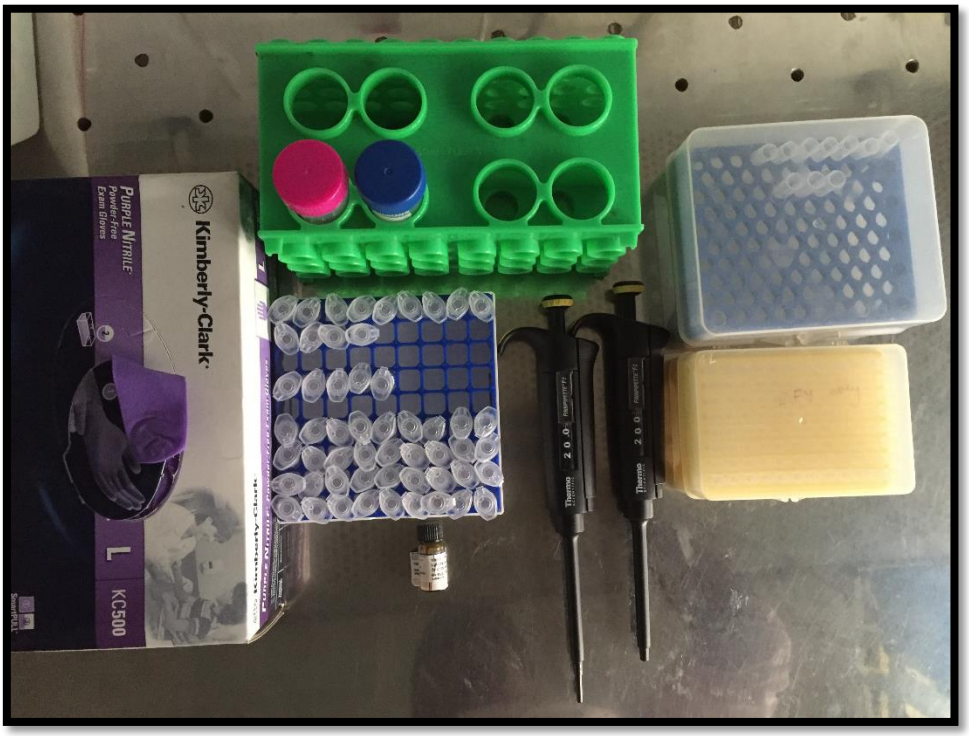
Photograph 3: Armamentarium for sampling

Photograph 4: Paper points for subgingival plaque sample collection

Photograph 5: Subgingival plaque sample collection



Photograph 6: Armamentarium for DNA extraction



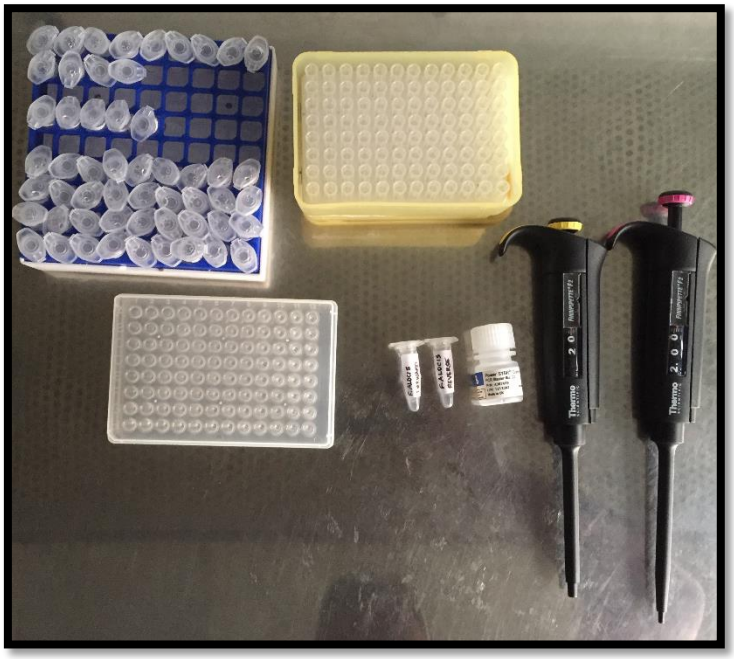
Photograph 7: Micropipettes and Tips

Photograph 8: DNA Isolation

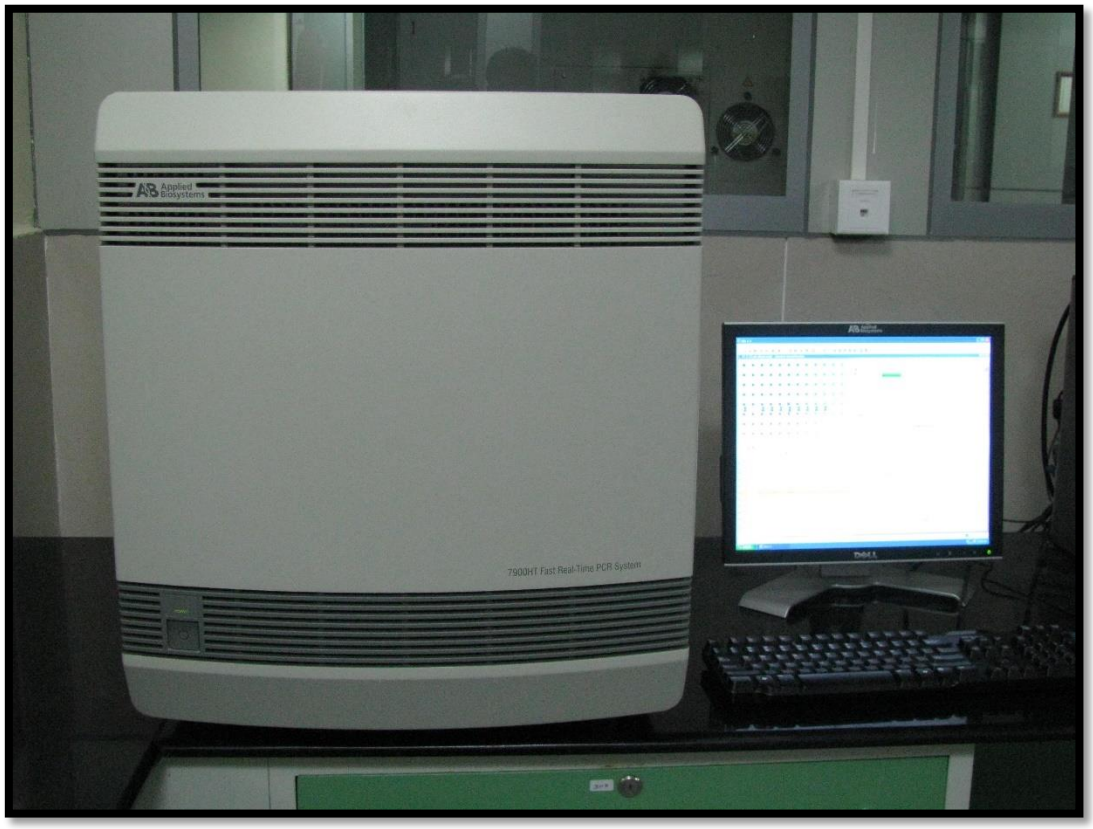
Photograph 9: Vortex

Photograph 10: Centrifuge

Photograph 11: Armamentarium for Real-Time PCR



Photograph 12: Real-Time PCR System



Statistical Analysis

STATISTICAL ANALYSIS

The statistical analysis was done using the computer software program SPSS version 20.0 (Statistical package for social science). Data is expressed as mean ± standard deviation of the parameters evaluated. All the data were analyzed using ANOVA test. In the statistical tool, the probability value $p \leq 0.05$ was considered as significant.

p value:

Two hypotheses were set in the beginning. The first is the null hypothesis, which assumes that the mean of two paired samples are equal. The second hypothesis is an alternative hypothesis, which assumes that the means of two paired samples are not equal. The level of significance was set at 5%. The p value or calculated probability was the estimated probability of rejecting the null hypothesis of a study question when that hypothesis was true. The smaller the p-value, the more significant the result was said to be. Differences between the two populations were considered significant when $p \leq 0.05$.

Results

RESULTS

The present clinical study was designed to identify *Filifactor alocis* (*F. alocis*) in periodontal biofilms using real-time polymerase chain reaction (RT-PCR). The clinical parameters (plaque index, sulcus bleeding index, probing pocket depth and the clinical attachment loss) and microbial parameters (CT values of *F. alocis*) were compared in three groups namely healthy control group, generalized chronic periodontitis (GCP) and generalized aggressive periodontitis (GAP).

Forty five subjects in the age group between 20 to 50 years were included in the study and their data were included in the statistical analysis.

The observations and results of clinical parameters and microbial parameters of the 45 subjects are summarized in the tables and figures.

Table –I: Comparison of mean scores in whole mouth plaque index in three groups

Table –II: Comparison of mean scores in selected site plaque index in three groups

Table –III: Comparison of mean scores in whole mouth bleeding index in three groups

Table –IV: Comparison of mean scores in selected site bleeding index in three groups

Table –V: Comparison of mean scores in whole mouth probing pocket depth in three groups in mm

Table –VI: Comparison of mean scores in selected site probing pocket depth in three groups in mm

Table –VII: Comparison of mean scores in whole mouth clinical attachment level in three groups in mm

Table –VIII: Comparison of mean scores in selected site clinical attachment level in three groups in mm

Table –IX: Comparison of mean CT scores of *Filifactor alocis* in selected site in three groups

Table –X: One way analysis comparison among three groups for various clinical and microbial parameters and their significance

Clinical Parameters

1. **Plaque index:**

Whole mouth:

The mean plaque index score for healthy group was 0.71±0.17, for chronic periodontitis group was 2.59±0.15, for aggressive periodontitis was 0.83±0.22.

Selected site:

The mean plaque index score for healthy group was 0.67±0.16, for chronic periodontitis group was 2.60±0.20, for aggressive periodontitis was 0.84±0.22.

Comparison of plaque index scores between three groups:

Whole mouth:

The mean difference of plaque index score between healthy control and chronic periodontitis was statistically highly significant ($p < 0.00001$).

Healthy control and aggressive periodontitis mean difference was statistically not significant ($p = 0.103965$).

Chronic periodontitis and aggressive periodontitis mean difference was statistically highly significant ($p < 0.00001$).

Selected site:

The mean difference of plaque index score between healthy control and chronic periodontitis was statistically highly significant ($p < 0.00001$).

Healthy control and aggressive periodontitis mean difference was statistically significant ($p = 0.02664$).

Chronic periodontitis and aggressive periodontitis mean difference was statistically highly significant ($p < 0.00001$).

2. **Sulcus bleeding index:**

Whole mouth:

The mean Sulcus bleeding index score for healthy group was 0.74±0.16, for chronic periodontitis group was 3.71±0.28, for aggressive periodontitis was 4.11±0.26.

Selected site:

The mean Sulcus bleeding index score for healthy group was 0.51±0.18, for chronic periodontitis group was 3.89±0.19, for aggressive periodontitis was 4.01±0.21.

Comparison of Sulcus bleeding index scores between three groups:

Whole mouth:

The mean difference of Sulcus bleeding index score between healthy control and chronic periodontitis was statistically highly significant ($p < 0.00001$).

Healthy control and aggressive periodontitis mean difference was statistically significant ($p < 0.00001$).

Chronic periodontitis and aggressive periodontitis mean difference was statistically significant (p = 0. 000318).

Selected site:

The mean difference of Sulcus bleeding index score between healthy control and chronic periodontitis was statistically significant (p < 0. 00001).

Healthy control and aggressive periodontitis mean difference was statistically significant (p < 0. 00001).

Chronic periodontitis and aggressive periodontitis mean difference was statistically not significant (p = 0.131701).

3. **Probing pocket depth:**

Whole mouth:

The mean Probing pocket depth for healthy group was 2.73±0.21 mm, for chronic periodontitis group was 5.92±0.35 mm, and for aggressive periodontitis was 6.23±0.45 mm.

Selected site:

The mean Probing pocket depth for healthy group was 2.71±0.22 mm, for chronic periodontitis group was 5.76±0.58 mm, and for aggressive periodontitis was 6.27±0.14 mm.

Comparison of Probing pocket depth between three groups:

Whole mouth:

The mean difference of Probing pocket depth between healthy control and chronic periodontitis was statistically highly significant ($p < 0.00001$).

Healthy control and aggressive periodontitis mean difference was statistically highly significant ($p < 0.00001$).

Chronic periodontitis and aggressive periodontitis mean difference was statistically significant ($p = 0.043077$).

Selected site:

The mean difference of Probing pocket depth between healthy control and chronic periodontitis was statistically highly significant ($p < 0.00001$).

Healthy control and aggressive periodontitis mean difference was statistically highly significant ($p < 0.00001$).

Chronic periodontitis and aggressive periodontitis mean difference was statistically significant ($p = 0.002494$).

4. **Clinical attachment level:**

Whole mouth:

The mean Clinical attachment level for healthy group was 0±0 mm, for chronic periodontitis group was 5.78±1.22 mm, and for aggressive periodontitis was 6.27±0.58 mm.

Selected site:

The mean Clinical attachment level for healthy group was 0±0 mm, for chronic periodontitis group was 6.18±0.46 mm, and for aggressive periodontitis was 6.25±0.16 mm.

Comparison of Clinical attachment level between three groups:

Whole mouth:

The mean difference of clinical attachment level between healthy control and chronic periodontitis was statistically highly significant ($p < 0.00001$).

Healthy control and aggressive periodontitis mean difference was statistically highly significant ($p < 0.00001$).

Chronic periodontitis and aggressive periodontitis mean difference was statistically not significant ($p = 0.176072$).

Selected site:

The mean difference of Clinical attachment level between healthy control and chronic periodontitis was statistically highly significant ($p < 0.00001$).

Healthy control and aggressive periodontitis mean difference was statistically highly significant ($p < 0.00001$).

Chronic periodontitis and aggressive periodontitis mean difference was statistically not significant ($p = 0.58559$).

Microbial Parameters

In the present study the CT values of *F. alocis* was analysed from the samples of the three groups of patients. The CT (Cycle Threshold) is defined as the number of cycles required for the fluorescent signal to cross the threshold (i.e. exceeds background level).

CT levels are inversely proportional to the amount of target nucleic acid in the sample. (i.e. the lower the CT level the greater the amount of target nucleic acid in the sample).

CTs < 29 are strong positive reactions indicative of abundant target nucleic acid in the sample

CTs of 30-37 are positive reactions indicative of moderate amounts of target nucleic acid

CTs of 38-40 are weak reactions indicative of minimal amounts of target nucleic acid which could represent an infection state or environmental contamination.

More CT value means less Bacterial load and less CT value means more Bacterial load

The mean CT value of healthy control group was 38.01 ± 1.87

The mean CT value of chronic periodontitis group was 30.85 ± 1.83

The mean CT value of aggressive periodontitis group was 28.92 ± 2.54

Comparison of mean CT values of three groups:

The mean difference of CT values between healthy control and chronic periodontitis was statistically significant ($p < 0.00001$).

The mean difference of CT values between healthy control and aggressive periodontitis was statistically significant ($p < 0.00001$).

The mean difference of CT values between chronic and aggressive periodontitis was statistically significant ($p = 0.00133$).

In comparison with generalized chronic periodontitis and generalized aggressive periodontitis groups, there is not much differences in bacterial load (though the bacterial load is moderate in both the groups based on CT value). While in comparison with the healthy control, there is a more difference in the bacterial load (less bacteria in healthy group). This indicates that, this bacteria *(F. alocis)* is prone to be more in diseased group than the healthy control.

Table – I: Mean scores of whole mouth plaque index in three groups

Whole mouth plaque index		
Group	Mean	± SD
Healthy	0.71	0.17
Chronic periodontitis	2.59	0.15
Aggressive periodontitis	0.83	0.22

Table – II: Mean scores of selected site plaque index in three groups

Selected site plaque index		
Group	Mean	± SD
Healthy	0.67	0.16
Chronic periodontitis	2.60	0.20
Aggressive periodontitis	0.84	0.22

Table – III: Mean scores of whole mouth bleeding index in three groups

Whole mouth bleeding index		
Group	Mean	± SD
Healthy	0.74	0.16
Chronic periodontitis	3.71	0.28
Aggressive periodontitis	4.11	0.26

Table – IV: Mean scores of selected site bleeding index in three groups

Selected site bleeding index		
Group	Mean	± SD
Healthy	0.51	0.18
Chronic periodontitis	3.89	0.19
Aggressive periodontitis	4.01	0.21

Table – V: Mean scores of whole mouth probing pocket depth in three groups in mm

	Whole mouth probing pocket depth	
Group	Mean	± SD
Healthy	2.73	0.21
Chronic periodontitis	5.92	0.35
Aggressive periodontitis	6.23	0.45

Table – VI: Mean scores of selected site probing pocket depth in three groups in mm

	Selected site probing pocket depth	
Group	Mean	± SD
Healthy	2.71	0.22
Chronic periodontitis	5.76	0.58
Aggressive periodontitis	6.27	0.14

Table–VII: Mean scores of whole mouth clinical attachment level in three groups in mm

	Whole mouth clinical attachment level	
Group	Mean	± SD
Healthy	0	0
Chronic periodontitis	5.78	1.22
Aggressive periodontitis	6.27	0.58

Table–VIII: Mean scores of selected site clinical attachment level in three groups in mm

	Selected site clinical attachment level	
Group	Mean	± SD
Healthy	0	0
Chronic periodontitis	6.18	0.46
Aggressive periodontitis	6.25	0.16

Table – IX: Mean scores of CT values in three groups

Group	Mean	SD
Healthy	38.01	1.87
Chronic periodontitis	30.85	1.83
Aggressive periodontitis	28.92	2.54

Table – X: One way analysis of comparison among three different groups

	Sum of squares	**df**	**Mean Square**	**F**	**Significance**
Plaque index (whole mouth)	32.9898	2	16.4949	499.12488	<0.00001*
Plaque index (selected site)	34.1871	2	17.0936	436.34279	<0.00001*
Bleeding index (whole mouth)	101.9004	2	50.9502	910.34146	<0.00001*
Bleeding index (selected site)	118.2031	2	59.1016	1575.04146	<0.00001*
Probing pocket depth (whole mouth)	112.9172	2	56.4586	469.30279	<0.00001*
Probing pocket depth (selected site)	111.5848	2	55.7924	417.17941	<0.00001*
Clinical attachment level (whole mouth)	365.0541	2	182.527	298.59329	<0.00001*
Clinical attachment level (selected site)	386.2569	2	193.1284	2444.88971	<0.00001*
Mean CT values	1377.1851	2	688.5926	155.29468	<0.00001*

* Significant

Plaque index (whole mouth)	**Sum of squares**	**df**	**Mean Square**	**F**	**Significance**
Healthy and chronic	26.3203	1	26.3203	1031.20709	<0.00001*
Healthy and aggressive	0.108	1	0.108	2.82441	0.103965
Chronic and aggressive	23.0563	1	23.0563	651.65	<0.00001*

* Significant

Plaque index (selected site)	Sum of squares	df	Mean Square	F	Significance
Healthy and chronic	27.8403	1	27.8403	821.13343	<0.00001*
Healthy and aggressive	0.2083	1	0.2083	5.47559	0. 02664*
Chronic and aggressive	23.232	1	23.232	509.7931	<0.00001*

* Significant

Bleeding index (whole mouth)	Sum of squares	df	Mean Square	F	Significance
Healthy and chronic	66.3053	1	66.3053	1295.26698	<0.00001*
Healthy and aggressive	85.3453	1	85.3453	1876.70367	<0.00001*
Chronic and aggressive	1.2	1	1.2	16.84492	0.000318*

* Significant

Bleeding index (selected site)	Sum of squares	df	Mean Square	F	Significance
Healthy and chronic	85.683	1	85.683	2534.28592	<0.00001*
Healthy and aggressive	91.5253	1	91.5253	2358.32147	<0.00001*
Chronic and aggressive	0.0963	1	0.0963	2.4112	0.131701

* Significant

Probing pocket depth (whole mouth)	Sum of squares	df	Mean Square	F	Significance
Healthy and chronic	76.5762	1	76.5762	945.91302	<0.00001*
Healthy and aggressive	92.0851	1	92.0851	762.0327	<0.00001*
Chronic and aggressive	0.7146	1	0.7146	4.49091	0.043077*

* Significant

Probing pocket depth (selected site)	Sum of squares	df	Mean Square	F	Significance
Healthy and chronic	70.1047	1	70.1047	366.66527	<0.00001*
Healthy and aggressive	95.337	1	95.337	2750.89968	<0.00001*
Chronic and aggressive	1.9355	1	1.9355	11.03721	0.002494*

* Significant

Clinical attachment level (whole mouth)	Sum of squares	df	Mean Square	F	Significance
Healthy and chronic	250.9678	1	250.9678	334.72293	<0.00001*
Healthy and aggressive	294.8468	1	294.8468	1763.88963	<0.00001*
Chronic and aggressive	1.7666	1	1.7666	1.92665	0.176072

* Significant

Clinical attachment level (selected site)	Sum of squares	df	Mean Square	F	Significance
Healthy and chronic	286.443	1	286.443	2696.11537	<0.00001*
Healthy and aggressive	292.9063	1	292.9063	23918.15266	<0.00001*
Chronic and aggressive	0.0361	1	0.0361	0.30428	0.58559

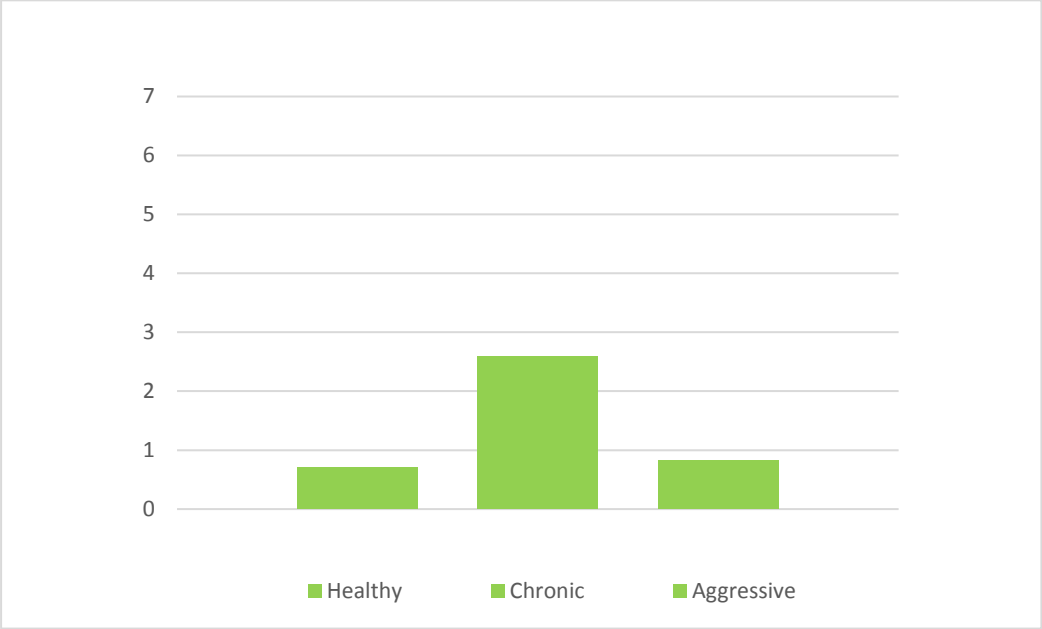
* Significant

CT Values	Sum of squares	df	Mean Square	F	Significance
Healthy and chronic	770.13	1	770.13	224.03161	<0.00001*
Healthy and aggressive	1239.967	1	1239.967	249.46893	<0.00001*
Chronic and aggressive	55.6807	1	55.6807	11.37667	0.00133*

* Significant

Figure 1: Comparison of mean scores of plaque index in three groups

WHOLE MOUTH



SELECTED SITE

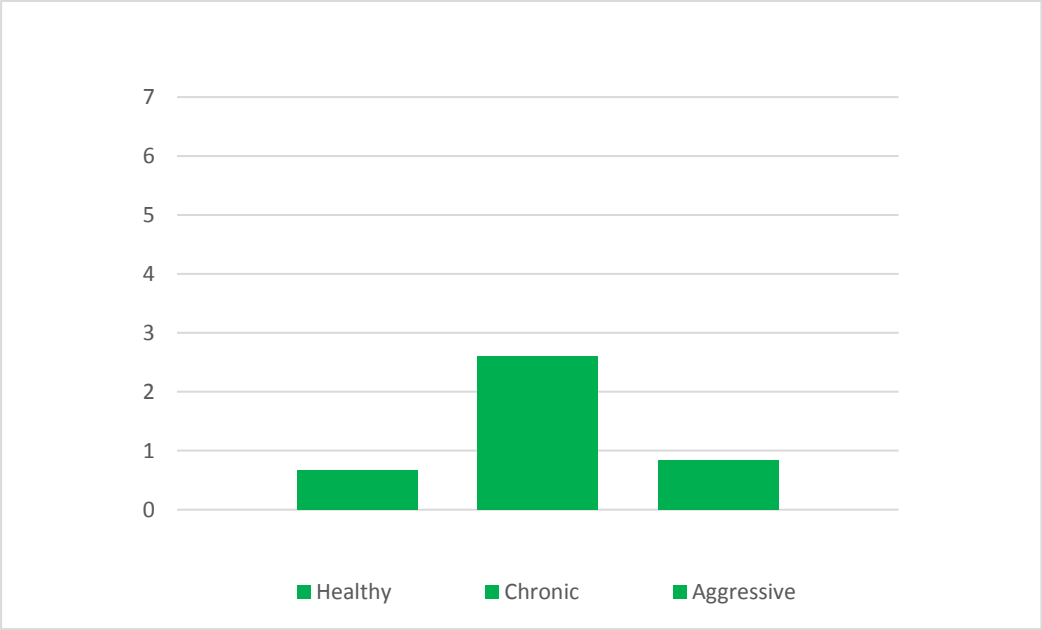
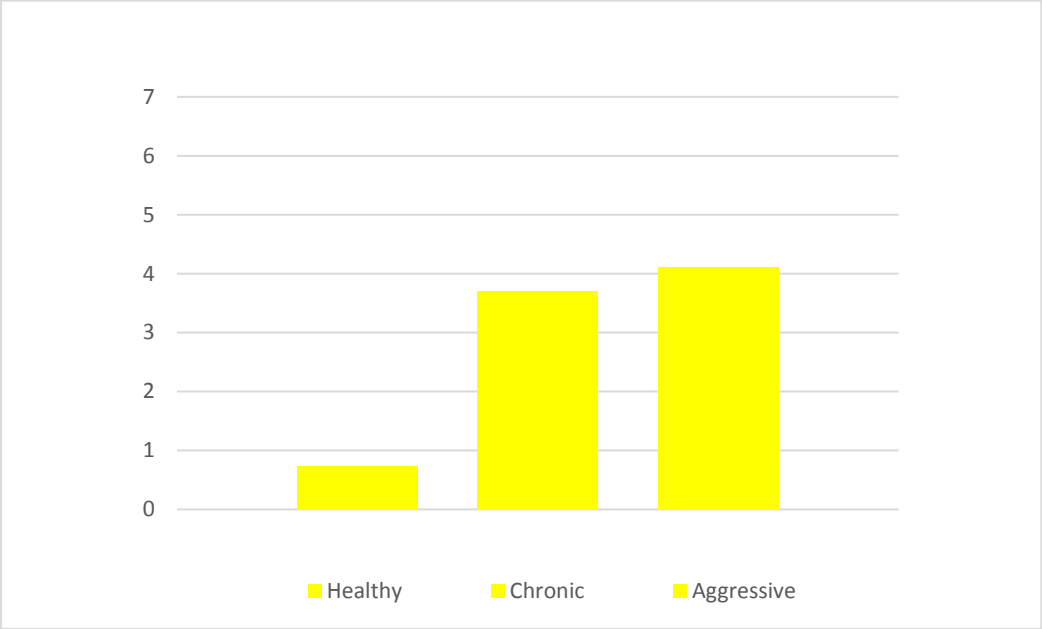


Figure 2: Comparison of mean scores of bleeding index in three groups

WHOLE MOUTH



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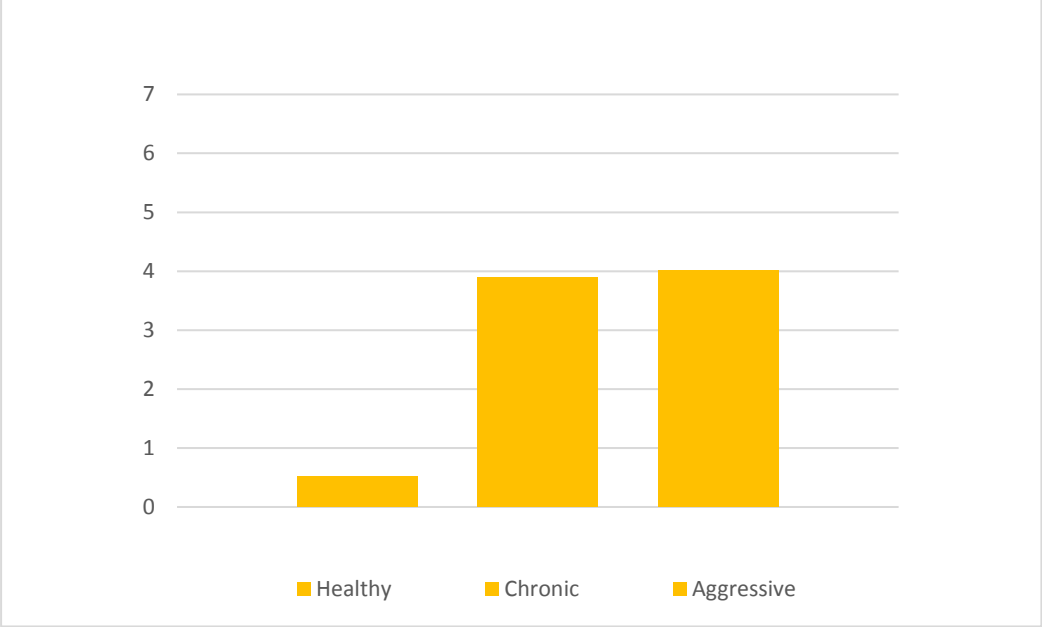
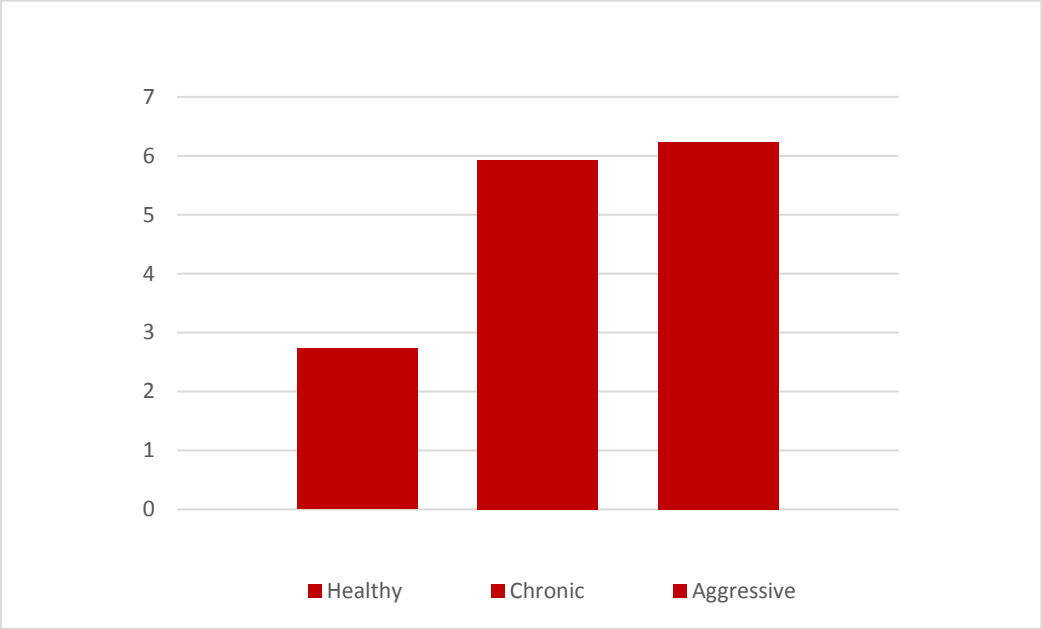


Figure 3: Comparison of mean scores of probing pocket depth in three groups

WHOLE MOUTH



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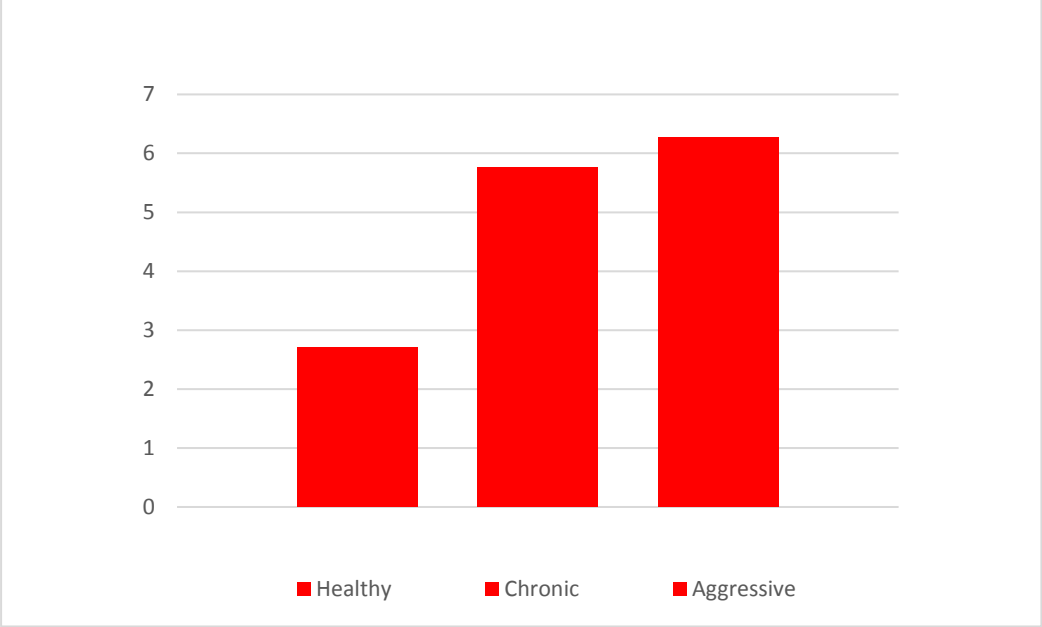
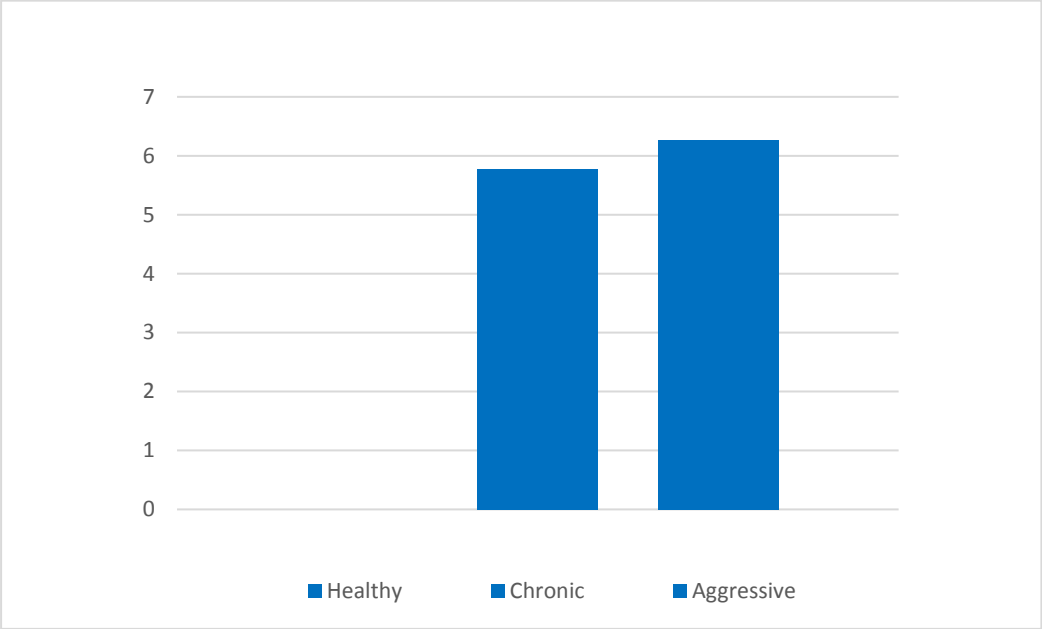


Figure 4: Comparison of mean scores of clinical attachment level in three groups

WHOLE MOUTH



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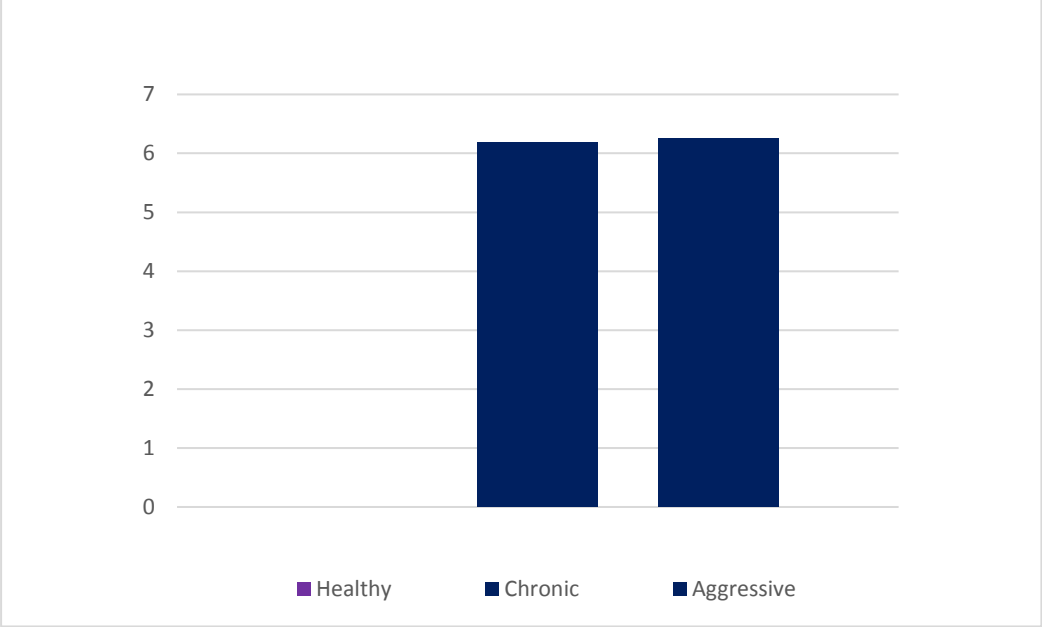
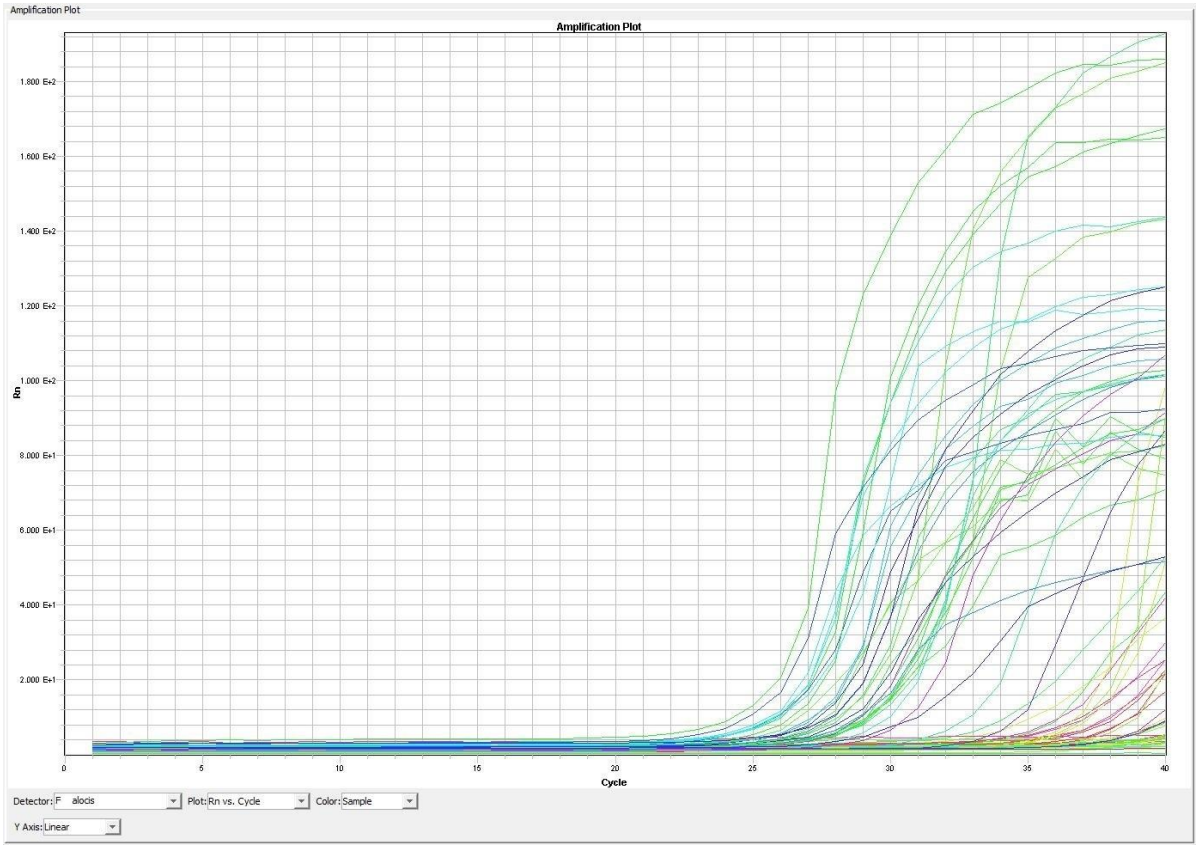


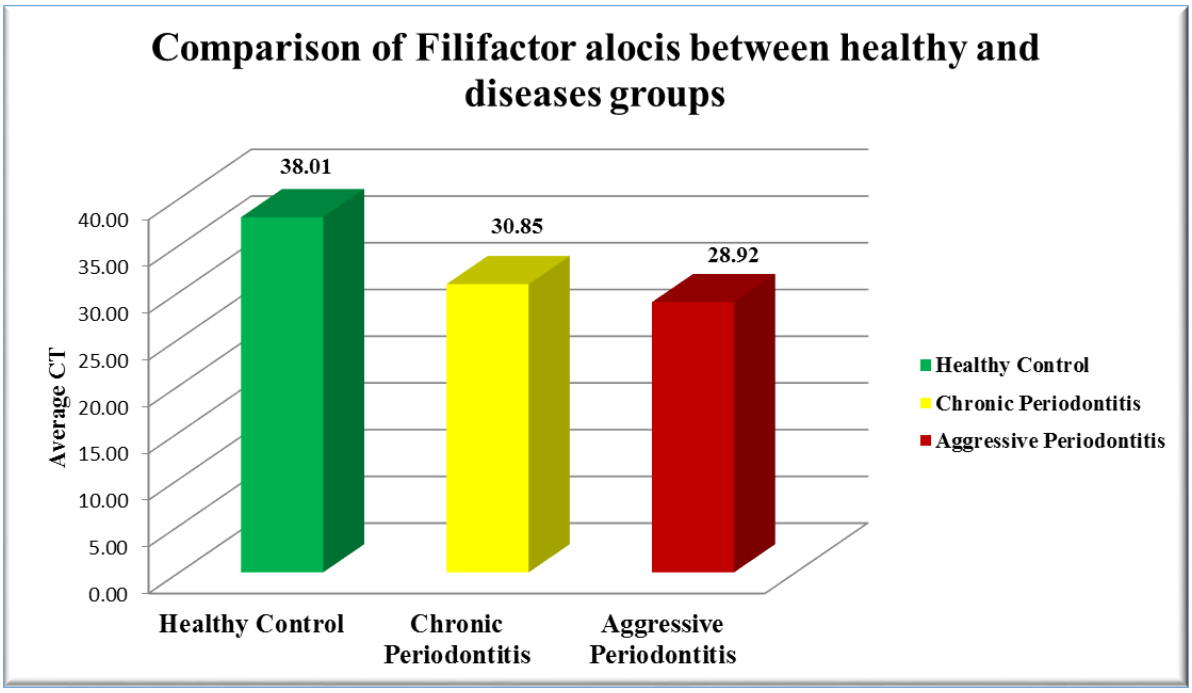
Figure 5: Real time PCR results – Amplification Plot (Rn Vs Cycle)



X Axis: Cycle (0, 5, 10, 15, 20, 25, 30, 35, 40)

Y Axis: Rn (0,2.0E+1,4.0E+1,6.0E+1,8.0E+1,1.0E+2,1.2E+2,1.4E+2,1.6E+2,1.8E+2)

Figure 6: Real time PCR results - Comparison of average CT values of samples in three groups



Discussion

DISCUSSION

Periodontitis is a ubiquitous microbial disease instigated by multifaceted biologic mechanisms between putative periodontal pathogens and the susceptible host leading to the devastation of periodontal tissues. Identification of the key pathogen opens the gateway for the successful treatment and prediction of prognosis of the periodontal disease.

The flora in periodontal pocket is characterized by the presence of innumerable phylotypes of bacteria of varied characteristics and more than 50% of them are not-yet cultured. It is possible that the traditional prototype of a principally gram negative flora concomitant with disease may undergo radical changes when more sophisticated diagnostic methods are used to validate this not-yet-cultured bacteria. This alters the therapeutic modality of treating the periodontal disease, which may lead to a more tangible benefit for the patient.

Filifactor alocis (*F. alocis*) is a new emerging periodontal pathogen which is a gram-positive anaerobic rod, fastidious in nature. It has recently gained reputation in the field of periodontology due to its documented evidence in pathogenesis of various forms of periodontal diseases such as chronic periodontitis, aggressive periodontitis, and periimplantitis by its inherent virulence characteristics. Both *invitro* and *invivo* studies have provided the evidence for *F. alocis* as a main periodontal pathogen.

The relevance of *F. alocis* is supported by several other epidemiological studies conducted in the past years using DNA-based techniques. *F. alocis* was detected in generalized aggressive periodontitis (GAP) patients as well as in chronic periodontitis (CP) patients with prevalence rates varying between 45% [***Hutter et al, 2003***][30] and 90% [***Dahlen G, Leonhardt A, 2006***] [9], liable on the methods employed. Some authors propose *F. alocis* as a marker organism for periodontal disease [***Dahlen, Leonhardt, 2006***] [9] and even for the change from periodontal health to disease [***Kumar PS, et al, 2006***] [4].

In this present study, Polymerase Chain Reaction (PCR) technique was selected for the identification of *F. alocis* in a total of forty five patients in the age group of 20-50 years reporting to the department of periodontics. The study subjects were selected based on their periodontal health status: subjects with healthy periodontium (n = 15), subjects with generalized chronic periodontitis (GCP) (n = 15) and subjects with generalized aggressive periodontitis (GAP) (n = 15).

Subjects were selected if they have a minimum of 20 natural teeth with at least 5 teeth in each quadrant. Subjects were considered healthy if the probing pocket depth (PPD) is less than or equal to 3mm. A subject was classified as having chronic periodontitis if presented with at least two sites per quadrant with probing pocket depth ≥ 4mm. Generalised aggressive periodontitis was diagnosed if the subjects were ≤ 25 years with a minimum of two interproximal sites per quadrant with probing pocket depth ≥ 4 mm and subjects 26 - 35 years with a minimum of two interproximal sites per quadrant with probing pocket depth ≥ 6 mm as per the criteria suggested by ***Demmer and Papapanaou, 2010*** [99].

Clinically, subjects were examined using clinical parameters namely Sulcus Bleeding Index, Plaque Index, Probing Pocket Depth and Clinical Attachment Level. In this study, evaluation of gingival bleeding was done using five scores by Sulcus Bleeding Index as it was a preferred index over dichotomous scoring.

Periodontal pathogens perform an imperative role in aetiology and pathogenesis of periodontitis. Although they are not the only determining factor, their absenteeism in the periodontal pocket stipulates more permanence and better prognosis of the patient [***Haffajee and Socransky, 2008***][17]. The periodontal pocket is the apt niche for pathogenic organisms to dwell, as is for *F. alocis*. Hence in this study the subgingival plaque biofilm was used to study the existence of the organism *F. alocis* in three groups namely healthy, generalised chronic periodontitis (GCP), and generalised aggressive periodontitis (GAP).

Before sampling, a sterile curette was used to remove the supragingival plaque and a sterile cotton roll was used to dry the sample site. Sampling of the selected sites was accomplished prior to mechanical treatment of the pocket. A pair of sterile forceps was used to introduce two sterile paper points (Densply®) (No. 30) in the pocket until resistance was felt and kept in place for 30 seconds in accordance to the studies by ***Schlafer et al., 2010[42], Bonner M et al,* 2014[100], *Trim RD et al, 2011[101].***

As discussed by Tanner and Goodson, there are many ways of collecting the subgingival plaque samples like curettes, scalers, paper points, barbed broaches within cannulas, irrigation of periodontal pockets, automatic pipette etc. [***Tanner and Goodson, 1986]*** [55] . In our present study we used paper points for obtaining the plaque samples as suggested by ***Monika Jusko et al, 2016[102], Spooner R. et al, 2016[103], Schlafer et al., 2010[42], Kumar PS et al, 2006[4],*** for collection of subgingival plaque in periodontal pockets for demonstrating putative periodontal pathogens including *F. alocis.*

The sample was taken from the single site per patient for the selected site analysis as it gives more relevant information about each site than pooled sample and first molar tooth has been selected for sampling in all subjects. Paper points retrieved from the site were immediately inserted in a sterile micro-centrifuge tube and kept over ice and transported to the laboratory for microbial analysis.

For the present study, PCR technique was employed for microbial analysis. More interestingly, PCR has also unveiled a number of uncultured and un-identified periodontal pathogens in health and disease in many epidemiologic studies [***Wecke, J., et al, 2000, Leys, E.J., et al., 2002, Griffen, A.L., et al., 2012***][104,105,106]

The gold standard traditional culture methods have characteristic advantages, but have limitations which includes the need to maintain bacterial vitality, the inability to detect low numbers of microorganisms with a detection limit ranging from 10^3 to 10^4 bacterial cells, labour intensiveness, requisite for skilled personnel, stringent sampling, transport environments and a protracted period of time for obtaining results [***Armitage, 1996***][19].

Other microbiological diagnostic tests such as dark field microscopy are limited to the detection of only motile periodontal pathogen, and immunodiagnostic methods like immunofluorescence assay, flow cytometry, etc., and enzymatic assays may can also show false positive results and cross-reactions [***Sanz et al., 2012***]**[107].**

PCR overwhelms the above restrictions and is capable of sensing even one copy of the sought DNA targets from the subjected clinical microbiologic samples collected for the study [***Sanz et al., 2004***]**[108].**

A PCR assay for the identification of *A. actinomycetemcomitans, P. gingivalis, P. intermedia, T. forsythensis and T. denticola* has recently been described [***Eick and Pfister, 2002***][11]. The authors advocated this test as a highly sensitive and specific means for the study of the subgingival plaque.

Schlafer et al, compared the prevalence of *F. alocis* in patients suffering from generalized aggressive periodontitis (GAP), chronic periodontitis (CP), and control subjects resistant to periodontitis through PCR followed by dot blot hybridization.[***Schlafer et al, 2010***][42]

Kumar PS et al, employed 16S PCR amplification using universal 16S primers of dental plaque samples, followed by cloning and sequencing to allow an open-ended and quantitative exploration of the bacterial populations present in periodontal health and disease [***Kumar PS et al, 2006***][4]

In the total of 45 subjects in our study, from whom the samples were taken, *F. alocis* was detected in all subjects through Real-time Polymerase Chain Reaction. But comparatively the level of the organism was significantly higher in generalized aggressive periodontitis patients whose mean probing pocket depth and mean bleeding index is significantly higher than the generalized chronic periodontitis group and the healthy control group.

Here in this study, the bacterial load is assessed by means of the cycle threshold (CT) values. More the CT value, less the microbial load. The mean CT value of healthy control group was 38.01 which is the highest among the three groups which indicate that the bacterial load is comparatively lower in healthy sites i.e. sites with probing depth of less than 3 mm which was used as a criteria for selection of healthy control group. The mean CT value of chronic periodontitis group was 30.85 and the mean CT value of aggressive periodontitis group was 28.92 which indicates the increasing level of microbial load in disease state.

The detection of the organism in the generalized aggressive periodontitis patients and in those suffering from generalized chronic periodontitis is a substantial evidence that proposes an association of *F. alocis* in periodontal disease. Also important to note is the low prevalence of the organism in the healthy control group who had a probing depth of less than 3mm and were in healthy periodontal status without the aid of laborious therapeutic efforts.

One would comment that, a horde of factors including oral hygiene status, immune response and genetic background of the host would be responsible for the periodontal status of the healthy subjects, they should also consider that increased detection of an organism in the generalized aggressive periodontitis and generalized chronic periodontitis groups along with infrequent detection in healthy patients specifies a pathogenic rather than commensal behaviour of the identified organism *F. alocis.*

The mean probing depth for generalized aggressive periodontitis is 6.27±0.14 mm and for generalized chronic periodontitis is 5.76±0.58 mm and the difference between them is less significant. But this slightly higher probing depth has resulted in more microbial load in aggressive periodontitis subjects.

One can claim that deep diseased periodontal pockets possess increased quantities of bacteria and that any organism should be isolated more persistently from GCP patients (mean pocket depth: 5.92±0.35 mm) and GAP patients (6.23±0.45 mm) than from healthy control patients with probing pocket depth in the normal range (2.73±0.21 mm).

However, statistical analysis of periodontal pockets greater than 4 mm reveals a significantly higher prevalence of *F. alocis* in both the GAP and the GCP group compared to the healthy control group. Although an association between PPD and bacterial load cannot be denied, these outcomes of our study indicate that the influence of pocket depth does not overthrow the above-mentioned results.

Though this study was performed in a small number of subjects, the prevalence of *F. alocis* in both GAP and GCP patients was found to be elevated as compared to healthy control. The emerging hypothesis suggest that instead of a single organism in oral microbial flora, it is the oral microbiome which is found to be responsible for the pathogenesis of disease by disrupting the balance in the symbiotic relationship among the organisms.

The putative pathogens cause dysbiosis and the role of putative periodontal pathogen *F. alocis* causing dysbiosis need to be evaluated in future studies for preventive, predictive and personalized treatment, beneficial for patients. *F. alocis* thus seems to be a powerful diagnostic marker organism for periodontal disease and it should be considered an important periodontal pathogen warranting further research in a large scale.

Summary and Conclusion

SUMMARY AND CONCLUSION

Identification of *Filifactor alocis* in periodontal biofilms using polymerase chain reaction (PCR) technique have been carried out in 45 subjects classified into three groups namely healthy group (n=15), generalized chronic periodontitis (GCP) (n=15) and generalized aggressive periodontitis (GAP) (n=15).

The clinical parameters such as plaque index, sulcus bleeding index, probing pocket depth, clinical attachment level and the microbiological parameter *F.alocis* level by Real-time PCR were evaluated and the following conclusions were drawn:

1. *F.alocis* has been identified in all the samples but comparatively the level of the organism was significantly higher in GAP patients than the GCP group and the healthy control group and also it was higher in GCP group than the healthy group.
2. Detection of *F.alocis* in the GAP and GCP groups along with scarce detection in control patients indicates pathogenic rather than commensal behaviour.
3. *F. alocis* seems to be a powerful diagnostic marker organism for periodontal disease and it should be considered an important periodontal pathogen.

Within the limits of present study, it can be concluded that the putative pathogens cause the dysbiosis and the role of putative periodontal pathogen *F. alocis* causing dysbiosis need to be evaluated in future studies for preventive, predictive and personalized treatment, beneficial for patients.

Furthermore, long term clinical studies monitoring clinical, microbiological and immunological changes caused by *F. alocis* in large number of samples are required.

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Annexure

Real-time PCR results of samples obtained for healthy group

SDS 2.3 RQ Results	1.2				
Assay Type	RQ Study				
EmbeddedFile	SDS				
Operator					
ThermalCycleParams					
#	**Status**	**Sample**	**Detector**	**Task**	**Avg Ct**
1	Analyzed	H1	Filifactor alocis	Target	35.90
2	Analyzed	H1	Filifactor alocis	Target	35.78
3	Analyzed	H2	Filifactor alocis	Target	38.90
4	Analyzed	H2	Filifactor alocis	Target	38.12
5	Analyzed	H3	Filifactor alocis	Target	40.00
6	Analyzed	H3	Filifactor alocis	Target	40.00
7	Analyzed	H23	Filifactor alocis	Target	37.76
8	Analyzed	H23	Filifactor alocis	Target	37.84
9	Analyzed	H24	Filifactor alocis	Target	34.89
10	Analyzed	H24	Filifactor alocis	Target	34.75
11	Analyzed	H26	Filifactor alocis	Target	40.00
12	Analyzed	H26	Filifactor alocis	Target	40.00
13	Analyzed	H27	Filifactor alocis	Target	34.12
14	Analyzed	H27	Filifactor alocis	Target	34.45
15	Analyzed	H58	Filifactor alocis	Target	37.90
16	Analyzed	H58	Filifactor alocis	Target	37.18
17	Analyzed	H59	Filifactor alocis	Target	40.00
18	Analyzed	H59	Filifactor alocis	Target	40.00
19	Analyzed	H60	Filifactor alocis	Target	38.90
20	Analyzed	H60	Filifactor alocis	Target	38.65
21	Analyzed	H61	Filifactor alocis	Target	37.90
22	Analyzed	H61	Filifactor alocis	Target	37.13
23	Analyzed	H82	Filifactor alocis	Target	40.00
24	Analyzed	H82	Filifactor alocis	Target	40.00
25	Analyzed	H83	Filifactor alocis	Target	37.78
26	Analyzed	H83	Filifactor alocis	Target	37.89
27	Analyzed	H84	Filifactor alocis	Target	40.00
28	Analyzed	H84	Filifactor alocis	Target	40.00
29	Analyzed	H85	Filifactor alocis	Target	37.45
30	Analyzed	H85	Filifactor alocis	Target	37.15
				Avearage	**38.01**
41	Analyzed	NTC	Filifactor alocis	Target	Undetermined
42	Analyzed	NTC	Filifactor alocis	Target	Undetermined

Real-time PCR results of samples obtained for generalized chronic periodontitis group

SDS 2.3 RQ Results	1.2				
Assay Type	RQ Study				
EmbeddedFile	SDS				
Operator					
ThermalCycleParams					
#	**Status**	**Sample**	**Detector**	**Task**	**Avg Ct**
1	Analyzed	CP8	Filifactor alocis	Target	31.91
2	Analyzed	CP8	Filifactor alocis	Target	31.85
3	Analyzed	CP9	Filifactor alocis	Target	33.12
4	Analyzed	CP9	Filifactor alocis	Target	33.25
5	Analyzed	CP10	Filifactor alocis	Target	30.02
6	Analyzed	CP10	Filifactor alocis	Target	30.07
7	Analyzed	CP12	Filifactor alocis	Target	27.45
8	Analyzed	CP12	Filifactor alocis	Target	27.78
9	Analyzed	CP13	Filifactor alocis	Target	29.15
10	Analyzed	CP13	Filifactor alocis	Target	29.35
11	Analyzed	CP14	Filifactor alocis	Target	31.01
12	Analyzed	CP14	Filifactor alocis	Target	31.12
13	Analyzed	CP25	Filifactor alocis	Target	32.97
14	Analyzed	CP25	Filifactor alocis	Target	32.37
15	Analyzed	CP34	Filifactor alocis	Target	30.89
16	Analyzed	CP34	Filifactor alocis	Target	30.75
17	Analyzed	CP35	Filifactor alocis	Target	32.90
18	Analyzed	CP35	Filifactor alocis	Target	33.05
19	Analyzed	CP38	Filifactor alocis	Target	31.90
20	Analyzed	CP38	Filifactor alocis	Target	31.89
21	Analyzed	CP39	Filifactor alocis	Target	28.05
22	Analyzed	CP39	Filifactor alocis	Target	27.85
23	Analyzed	CP40	Filifactor alocis	Target	30.01
24	Analyzed	CP40	Filifactor alocis	Target	30.14
25	Analyzed	CP73	Filifactor alocis	Target	32.96
26	Analyzed	CP73	Filifactor alocis	Target	32.78
27	Analyzed	CP68	Filifactor alocis	Target	31.90
28	Analyzed	CP68	Filifactor alocis	Target	31.87
29	Analyzed	CP69	Filifactor alocis	Target	28.67
30	Analyzed	CP69	Filifactor alocis	Target	28.45
				Average	**30.85**

Real-time PCR results of samples obtained for generalized aggressive periodontitis group

SDS 2.3 RQ Results	1.2				
Assay Type	RQ Study				
EmbeddedFile	SDS				
Operator					
ThermalCycleParams					
#	**Status**	**Sample**	**Detector**	**Task**	**Avg Ct**
1	Analyzed	AG19	Filifactor alocis	Target	30.60
2	Analyzed	AG19	Filifactor alocis	Target	30.26
3	Analyzed	AG20	Filifactor alocis	Target	26.45
4	Analyzed	AG20	Filifactor alocis	Target	26.58
5	Analyzed	AG21	Filifactor alocis	Target	28.90
6	Analyzed	AG21	Filifactor alocis	Target	28.76
7	Analyzed	AG22	Filifactor alocis	Target	25.56
8	Analyzed	AG22	Filifactor alocis	Target	25.69
9	Analyzed	AG51	Filifactor alocis	Target	29.81
10	Analyzed	AG51	Filifactor alocis	Target	30.01
11	Analyzed	AG52	Filifactor alocis	Target	32.12
12	Analyzed	AG52	Filifactor alocis	Target	32.48
13	Analyzed	AG53	Filifactor alocis	Target	28.90
14	Analyzed	AG53	Filifactor alocis	Target	29.08
15	Analyzed	AG54	Filifactor alocis	Target	31.12
16	Analyzed	AG54	Filifactor alocis	Target	32.97
17	Analyzed	AG55	Filifactor alocis	Target	26.40
18	Analyzed	AG55	Filifactor alocis	Target	26.85
19	Analyzed	AG56	Filifactor alocis	Target	29.94
20	Analyzed	AG56	Filifactor alocis	Target	30.06
21	Analyzed	AG57	Filifactor alocis	Target	32.25
22	Analyzed	AG57	Filifactor alocis	Target	32.86
23	Analyzed	AG66	Filifactor alocis	Target	24.90
24	Analyzed	AG66	Filifactor alocis	Target	24.59
25	Analyzed	AG67	Filifactor alocis	Target	30.78
26	Analyzed	AG67	Filifactor alocis	Target	30.90
27	Analyzed	AG71	Filifactor alocis	Target	25.67
28	Analyzed	AG71	Filifactor alocis	Target	25.80
29	Analyzed	AG72	Filifactor alocis	Target	28.64
30	Analyzed	AG72	Filifactor alocis	Target	28.75
				Average	**28.92**

TAMIL NADU GOVERNMENT DENTAL COLLEGE AND HOSPITAL

DEPARTMENT OF PERIODONTICS

IDENTIFICATION OF FILIFACTOR ALOCIS IN PERIODONTAL BIOFILMS USING POLYMERASE CHAIN REACTION TECHNIQUE – A CROSS SECTIONAL STUDY

Proforma

Date : OP No.: S. No.:

Name : Age : Sex:

Occupation : Income:

Address : Phone Number:

CHIEF COMPLAINTS AND DURATION:

HISTORY OF PRESENT ILLNESS:

PAST MEDICAL HISTORY:

PAST DENTAL HISTORY:

FAMILY HISTORY:

PERSONAL HISTORY:

a) Oral Hygiene Practices :

b) Habits :

c) Menstrual History :

d) Menopause :

e) H/o. Stress Factor :

GENERAL EXAMINATION

a) Extra-Oral Examination

b) Examination of Lymphnodes

INTRA-ORAL EXAMINATION WITH CLINICAL FINDINGS:

Buccal mucosa:

Vestibule:

Hard palate:

Soft palate:

Tonsils:

Tongue:

Floor of the mouth:

Teeth:

Decayed

Missed

Filled teeth

Gingiva

Plaque index

18	17	16	15	14	13	12	11	21	22	23	24	25	26	27	28
48	47	46	45	44	43	42	41	31	32	33	34	35	36	37	38

Inference:

Bleeding Index

18	17	16	15	14	13	12	11	21	22	23	24	25	26	27	28
48	47	46	45	44	43	42	41	31	32	33	34	35	36	37	38

Inference:

Probing depth and attachment loss in millimetre

Maxillary:

CAL																
PPD																
	18	17	16	15	14	13	12	11	21	22	23	24	25	26	27	28
PPD																
CAL																

Mandibular:

CAL																
PPD																
	48	47	46	45	44	43	42	41	31	32	33	34	35	36	37	38
PPD																
CAL																

Inference:

Selected site clinical and microbial analysis

Parameters	**Tooth number**
PI	
BOP	
PPD	
CAL	
Sampling for Microbial analysis	

Investigations:

1. Biochemical / Haematological Investigation:

Haemoglobin	:
Total leucocyte count	:
Differential leucocyte count	:
Bleeding time	:
Clotting time	:
Erythrocyte sedimentation rate	:
Random blood sugar	:

2. Others :

Blood Pressure:

Test Dose for L.A:

RADIOGRAPHIC EVALUATION

Intra-Oral Periapical Radiograph/Orthopantomogram (IOPA/OPG)

PROVISIONAL DIAGNOSIS

PROGNOSIS

TREATMENT PLAN

FITNESS FOR TREATMENT

TREATMENT DONE

DATE : PROCEDURE : SIGNATURE :

SIGNATURE OF THE PROFESSOR

ஆராய்ச்சி பற்றிய தகவல் படிவம்

ஆராய்ச்சி மேற்கொள்பவர்: மருத்துவர். க. பூரணா

வழிநடத்துபவர்: மருத்துவர். மகேஸ்வரி ராஜேந்திரன் M. D. S.

ஆராய்ச்சி நிறுவனத்தின் பெயர்: தமிழ்நாடு அரசு பல் மருத்துவக் கல்லூரி மற்றும் மருத்துவமனை, சென்னை.

ஆராய்ச்சியின் தலைப்பு:
பாலிமரேஸ் தொடர்வினை நுட்பத்தை பயன்படுத்தி பல்லைச்சுற்றிய உயிர்த்திரைகளில் உள்ள ஃபில்லிஃபாக்டர் அலோசிஸ் நுண்ணுயிரி அடையாளம் கண்டறிதல்- ஒரு குறுக்கு வெட்டு ஆய்வு

ஆராய்ச்சியின் நோக்கம்:
பாலிமரேஸ் தொடர்வினை நுட்பத்தை பயன்படுத்தி பல்லைச்சுற்றிய உயிர்த்திரைகளில் உள்ள ஃபில்லிஃபாக்டர் அலோசிஸ் நுண்ணுயிரியல் மதிப்பீட்டு ஆய்வினை மேற்கொள்ளுதல்.

செய்முறை:
கீழ்கண்ட ஆய்வுகள்/பரிசோதனைகள் உங்களுக்காக செய்யப்படும்
பரிசோதனை:
வாய் பரிசோதனை: உட்புறம், வெளிப்புறம்
வழக்கமான இரத்தப் பரிசோதனை: உங்களின் கையிலிருந்து இந்தப் பரிசோதனைக்காக 5 மில்லியளவு (ஒரு மேஜைக் கரண்டி அளவு) இரத்தம் எடுக்கப்படும்.
நோயுற்ற பகுதியின் ஊடு கதிர்படம்.
ஒவ்வாமை ஏற்படுகிறதா என்பதைத் தெரிந்து கொள்ள 0.5 மில்லி 2% லிக்னோகெயின் மயக்க மருந்து உங்களின் கையில் பரிசோதனைக்காகக் கொடுக்கப்படும்.
காகிதமுனைகளை உபயோகித்து நோயுற்ற பல் உள்ஈறு மாதிரிகள் நுண்ணுயிரியல் பரிசோதனைக்காக எடுக்கப்படும்.
பின்பு, நோயுற்ற பகுதியில் மயக்க மருந்து கொடுக்கப்படும்.
அல்ட்ரா சோனிக் ஸ்கேலர் மற்றும் கைக் கருவிகள் பயன்படுத்தி பல் மற்றும் பல்லின் வேர் சுத்தம் செய்யப்படும். உப்புநீர் கொண்டு நோயுற்ற பகுதி சுத்தம் செய்யப்படும்.
மருத்துவ மதிப்பீடு, நுண்ணுயிரியல் மதிப்பீடு மேற்கொள்ளப்படும்

பங்கேற்பதினால் விளையும் நன்மைகள்:
உங்களின் நாள்பட்ட பல் ஈறு நோய்க்கு சிகிச்சை அளிக்கப்படும்.
பங்கேற்பதினால் வரக்கூடிய பக்க விளைவுகள்:
வலி, வீக்கம் மற்றும் பயன்படுத்தும் பொருட்களினாலும், மயக்க மருந்தினாலும் (2% லிக்னோகெயின்) சில நேரங்களில் ஒவ்வாமை ஏற்பட வாய்ப்புண்டு. அதற்காகத் தேவைப்படும் மருந்துகளும் மருத்துவமும் வழங்கப்படும்.

இரகசிய காப்பு:
உங்களை பற்றிய குறிப்புகள் பிறர் அறியா வண்ணம் ஆராய்ச்சி முடியும் வரை இரகசியமாக பாதுகாக்கப்படும். அதை வெளியிடும் நேரங்களில் எந்த தனி அடையாளங்களும் வெளிப்பட வாய்ப்பு கிடையாது.
தன்னார்வ பங்கேற்பு:
இந்த ஆராய்ச்சியில் பங்கு பெறுவது தங்களின் தனிப்பட்ட முடிவு மற்றும் இந்த ஆராய்ச்சியில் இருந்து நீங்கள் எப்போது வேண்டுமானாலும் விலகிக்கொள்ளலாம். தங்களின் இந்த திடீர் முடிவு உங்களுக்கோ அல்லது ஆராய்ச்சியாளருக்கோ எந்த வித பாதிப்பையும் ஏற்படுத்தாது என்பதை தெரியப்படுத்துகிறோம்.

நோயாளியின் பெயர் கையொப்பம்/கைரேகை

ஆராய்ச்சி தொடர்புடைய தகவல்களுக்கு
மரு. க. பூரணா
முதுநிலை பல் மருத்துவ மாணவர், பல் மற்றும் பல் புறத்திசுவியல் துறை
தமிழ்நாடு அரசு பல் மருத்துவக் கல்லூரி மற்றும் மருத்துவமனை, சென்னை

பங்கேற்பாளரின் உரிமை தொடர்புடைய தகவல்களுக்கு:
மரு.B.சரவணன், M.D.S., Ph.D.,
தலைவர்,
நிறுவன நெறிமுறைகள் குழு
தமிழ்நாடு அரசு பல் மருத்துவக் கல்லூரி மற்றும் மருத்துவமனை, சென்னை.

Participant Information Sheet

Investigator: Dr. K. Poorana **Guide:** Dr. Maheaswari Rajendran M.D.S

Title of the study: IDENTIFICATION of FILIFACTOR ALOCIS in PERIODONTAL BIOFILMS USING POLYMERASE CHAIN REACTION TECHNIQUE – A CROSS SECTIONAL STUDY

Name of the research institution: Tamil Nadu Government Dental College & Hospital,Chennai.

The investigator, Dr. K. Poorana under the guidance of Dr. Maheaswari Rajendran M.D.S is conducting a study as titled above with aim to identify the presence of *Filifactor alocis* in periodontal biofilms through PCR technique in healthy, generalized chronic periodontitis and generalized aggressive periodontitis subjects.

1. Procedure: The following examination/investigation will be done for you.

- Intra oral examination, Extra-oral examination
- Routine blood test – 5ml (1 table spoon) of blood will be drawn from your hand
- Routine standard X-ray will be taken with protection (lead apron , thyroid collars)
- Subgingival plaque sampling – using paper points samples from pockets in gums will be taken for microbiological analysis
- Microbiological evaluation will be performed

2. Risk of participation:

There may be allergic reaction to materials used in the study. Patient may experience pain, discomfort, swelling following the procedure, but due precautions will be taken to prevent as well as manage.

3. Benefits of participation

Treatment for your diseased status will be given for improving periodontal status.

4. Confidentiality:

The identity of the patients participating in the research will be kept confidential throughout the study. In the event of any publication or presentation resulting from the research, no personally identifiable information will be shared.

5. Participant's rights:

Taking part in the study is voluntary. You are free to decide whether to participate in the study or to withdraw at any time; your decision will not result in any loss of benefits to which you are otherwise entitled. The results of this study will be intimated to you at the end of the study period or during the study if anything is found abnormal which may aid in the management or treatment.

6. Compensation: Nil

7. Contacts:

For queries related to the study:

Primary Investigator: Dr. K. Poorana
Contact Details: Post Graduate student, Department of Periodontics
Tamilnadu Govt. Dental College & Hospital, Chennai -600003
Phone number: 9486767124

Contact details regarding rights of the participant:

Dr. B. Saravanan, M.D.S., Ph.D.,
The Chairperson,
Institutional Ethical committee
Tamilnadu Govt. Dental College & Hospital, Chennai

ஒப்புதல் படிவம்

ஆராய்ச்சியின் தலைப்பு

பாலிமரேஸ் தொடர்வினை நுட்பத்தை பயன்படுத்தி பல்லைச்சுற்றிய உயிர்த்திரைகளில் உள்ள ஃபில்லிஃபாக்டர் அலோசிஸ் நுண்ணுயிரி அடையாளம் கண்டறிதல்- ஒரு குறுக்கு வெட்டு ஆய்வு

பெயர் : புறநோயாளி எண்:

வயது/பால்: ஆராய்ச்சி சேர்க்கை எண்:

முகவரி :

தொலைபேசி :

நான் ..... வயது ..... என்னுடைய சுயநினைவுடனும் மற்றும் முழு சுதந்திரத்துடனும் இந்த மருத்துவ ஆராய்ச்சியில் என்னை சேர்த்துக்கொள்ள ஒப்புதல் அளிக்கிறேன்.

<u>கீழ்க்காணப்படும் நிபந்தனைகளுக்கு நான் சம்மதிக்கிறேன்:</u>

- நான் இந்த ஆராய்ச்சியின் நோக்கம் மற்றும் செயல்முறைகள் பற்றி முழுமையாக தெரிவிக்கப்பட்டுள்ளேன்.
- இந்த பரிசோதனைக்காக பற்கள் மற்றும் ஈறுகளில் சுத்தம் செய்யும் சிகிச்சை செய்ய வேண்டியுள்ளதாக அறிகிறேன்.
- என் உடல்நலம் பாதிக்கப்பட்டாலோ அல்லது எதிர்பாராத வழக்கத்திற்கு மாறான நோய் குறிகள் தென்பட்டாலோ அதனை விலக்குவதற்கும் முழு உரிமை உள்ளதாக அறிகிறேன்.
- நான் ஏற்கனவே உட்கொண்ட மற்றும் உட்கொள்கின்ற மருந்துகளின் விபரங்களை ஆராய்ச்சியாளரிடம் தெரிவித்துள்ளேன்.
- என் மருத்துவ குறிப்பேடுகளை இந்த ஆராய்ச்சியில் பயன்படுத்திக்கொள்ள சம்மதிக்கிறேன். இந்த ஆராய்ச்சி மையமும் ஆராய்ச்சியாளரும் என்னுடைய விபரங்கள் அனைத்தையும் இரகசியமாக வைப்பதாக அறிகிறேன்.

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நோயாளியின் பெயர்	கையொப்பம்	தேதி
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ஆராய்ச்சியாளரின் பெயர்	கையொப்பம்	தேதி

Informed Consent Form

IDENTIFICATION OF FILIFACTOR ALOCIS IN PERIODONTAL BIOFILMS USING POLYMERASE CHAIN REACTION TECHNIQUE – A CROSS SECTIONAL STUDY

Participant ID No:

"I have read the foregoing information, or it has been read to me. I have had the opportunity to ask questions about it and any questions I have asked have been answered to my satisfaction. I consent voluntarily to participate as a participant in this study and understand that I have the right to withdraw from the study at any time without in any way it affecting my further medical care."

Date	Name of the participant	Signature/thumb impression of the participant

[The literate witness selected by the participant must sign the informed consent form. The witness should not have any relationship with the research team; If the participant doesn't want to disclose his / her participation details to others, in view of respecting the wishes of the participant, he / she can be allowed to waive from the witness procedure (This is applicable to literate participant ONLY). This should be documented by the study staff by getting signature from the prospective participant]

"I have witnessed the accurate reading of the consent form to the potential participant and the individual has had opportunity to ask questions. I confirm that the individual has given consent freely"

Date	Name of the witness	Signature of the witness

Date	Name of the interviewer	Signature of the interviewer